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Progress was made this period on the important question of how FTI efficacy is achieved in breast cancer. Clinical trials have revealed that breast cancers respond to FTI but only in a minority of cases. What factors dictate FTI efficacy? In this period, we advanced our studies of the role of cyclin B1, a key regulator of mitosis, as a critical target for RhoB suppression in FTI-induced apoptosis. Evidence that cyclin B1 downregulation was critical for FTI-induced tumor suppression was obtained. Mechanistic studies revealed that at early times RhoB suppressed the transcription and the nuclear accumulation of cyclin B1 that occurs in cells destined to undergo FTI-induced cell death. In a second line of work, we discovered that Rho and Myc interact genetically in breast tumor formation. Specifically, we found that loss of RhoB accelerated breast tumor formation in MMTV-c-myc mice. Preliminary studies suggest that this effect may be based increased stability of the c-Myc protein, prompting the hypothesis that RhoB regulates the turnover of c-Myc in cancer cells. Our work furthers the notion that cyclin B1 is a critical proapoptotic target of RhoB and it reveals suggestive ineractions between RhoB and Myc in breast cancer formation.

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Introduction

We proposed to test the hypothesis that RhoB alteration is responsible for mediating FTI action malignant epithelial cells of the breast. Recent studies suggest that prooncogenic Rho proteins play an important role in driving breast cancer, for example, as in highly aggressive inflammatory breast cancers where overexpression of RhoC is a key oncogenic driver [1,2]. RhoB is an antioncogenic member of the Rho gene family which regulates cellular actin structure, adhesion, motility, proliferation, and survival [3]. RhoB may contribute to the regulation of a signaling cascade that mediates proliferation in response to epidermal growth factor (EGF) [4], a major mitogen for normal and neoplastic breast cells. Moreover, RhoB has been assigned a specialized role in the intracellular trafficking of the EGF receptor [5].

Recent work in our laboratory has identified RhoB as a key target for alteration by farnesyltransferase inhibitors (FTIs), an experimental class of cancer therapeutics that are being tested in clinical trials for breast cancer treatment [6]. In preclinical models, FTIs have displayed relatively unique properties: while largely nontoxic to normal cells they dramatically inhibit the proliferation and/or survival of neoplastically transformed cells. These 'cancer-selective' properties are of significant interest, in part because of they can be traced to molecules other than the molecule that was initially strategized as a target for FTIs, namely Ras [7]. Interestingly, several lines of genetic evidence that have been obtained strongly support a model in which that the antineoplastic properties of FTIs are mediated by a gain-of-function in the antioncogenic RhoB protein [8-12]. Although the main support has derived from mouse models, there is also more recent evidence that RhoB mediates the antineoplastic FTI response in human breast carcinoma cells or RhoC-transformed human mammary epithelial cells [11].

In this research period, we made progress on both aims of our proposal to address an clinically important question that has emerged with the finding that FTIs display efficacy in human breast cancer, namely, what are the factors which dictate efficacy in the minority of breast cancer patients that respond to FTI treatment? Aim 1

of the project was to test whether deletion of the RhoB gene compromised the antitumor response to FTI treatment in mouse models of human breast cancer. At earlier stages of the work this aim was stymied by mouse breeding issues that arose. In this period, we made progress in circumventing this block by changing the mouse model of breast cancer used. In Aim 2, which was refocused with the permission of the USAMRMC given progress on the original aim, we sought to define effector signals involved in mediating FTI-induced apoptosis, based on preclinical evidence that this process is important to in vivo efficacy in mice [10]. Recent progress supports a critical role for RhoB in regulating the level and subcellular localization of cyclin B1 in FTI-induced apoptosis. One point to confirm with regard to this project is that in 2002 the principle investigator was altered from Dr. Li to Dr. Kamasani due to personnel changes in the laboratory.

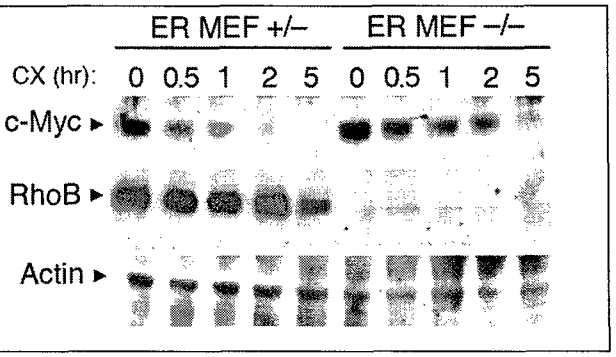
Body

Aim 1. This aim included mouse breeding experiments to move the null allele from a rhoB "knockout" (KO) mouse onto various 'oncomouse' models for breast cancer that use the mouse mammary tumor virus (MMTV) promoter to drive specific oncogene expression in the mammary gland. However, issues emerged with regard to breeding and tumor formation in the strains we originally planned to use. In this grant period, we were able to overcome this issue using MMTV-c-myc mice, which can be bred successfully to rhoB KO mice.

Interestingly, preliminary work in a small cohort of mice suggests that loss of one or two copies of rhoB gene elevates the incidence of c-myc-driven BRCA formation in this model. Specifically, both MMTV-c-myc;rhoB+/- and MMTV-c-myc;rhoB-/- virgin females exhibit tumor formation at 4-6 months of age, compared to MMTV-myc;rhoB+/+ virgins which remain tumor-free up to one year of age (data not shown). MMTV-c-myc;rhoB+/+ mice exhibit tumor formation at ~6 months of age when carried on a multiparous schedule (which elevates expression of the MMTV-c-myc transgene due to estrogen-dependent activation of the MMTV promoter). Therefore, rhoB loss phenocopied c-Myc elevation in terms of the kinetics of tumor formation. The simplest

explanation for this effect was that rhoB somehow influenced the expression of c-Myc. Strikingly, initial investigations of this possibility in our E1a/ras-transformed cell model revealed that the rhoB genotype markedly affected the halflife of c-Myc protein, which increased under conditions of rhoB loss (see Fig. 1).

Fig. 1. RhoB deletion stabilizes c-Myc protein. ER MEFs[10] with the rhoB genotypes indicated were treated various times with 10 μg/ml cycloheximide (CX) before cell extracts were prepared and 50 μg extract/lane was analyzed by Western blotting with antibodies to c-Myc (Santa Cruz Biotechnology), RhoB (Bethel), and Actin (Santa Cruz Biotechnology) as recommended by the vendor. Heterozygous cells were used to control for the neo cassette in null cells.



We are currently working to confirm this effect in c-myc-transformed mouse mammary cells where rhoB levels are varied by gene deletion or by expression of a rhoB-targeted RhoGAP protein (kindly provided by Y. Zheng, Univ. of Cincinatti School of Medicine). While we have yet to determine the molecular mechanism underlying this effect, it is intriguing to note the evidence that RhoB can promote maturation of endosomal vesicles toward the multivesicle body (H. Mellor, Univ. of Bristol, pers. comm.), which can deliver cargo to the lysosome for degradation, as well as the evidence that under conditions of overexpression, such as those found in breast cancer, c-Myc may be trafficked to the cytosol for degradation (W. Tansey, Cold Spring Harbor Laboratory, pers. comm.).

As we have shown previously, FTI can modulate RhoB, so the above results offer indirect support of the hypothesis that FTI may modulate breast tumor formation via RhoB. In the next grant period, we will determine whether c-myc-driven breast tumors formed in rhoB heterozygous or null backgrounds are indeed less susceptible to growth inhibition (perhaps due to elevated c-myc expression). We have recently initiated interactions with Johnson and Johnson, Inc., to test whether this effect may be seen using not only the Merck FTI that we have used but also another clinically tested FTI in the compound R115777.

Aim 2. In the last period, this Aim was refocused on new developments in work to define the determinants for FTI-induced apoptosis, which is known from mouse studies to be critical for drug efficacy [10]. The original Aim was to assess a possible role for PRK kinase – a key effector kinase for RhoB signaling - in the FTI response. Positive support for the hypothesis that RhoB and PRK mediate growth suppression by FTI in epithelial cells was obtained in the last period [11,12]. However, related studies suggested that PRK might not be important for FTI to trigger apoptosis. For this reason, we initiated efforts to define other RhoB effector molecules that may be important for apoptosis by FTI. Last year, these studies matured sufficiently to lead to two publications at *Oncogene* and *Cell Biology & Therapy* [13,14], preprints of which had been included in the last report. The USAMRMC approved our request to assess what we had identified as a candidate proapoptotic target of RhoB in cyclin B1, a key regulator of mitotic events. In this grant period, we have continued to analyze the role of cyclin B1 in the important question of what factors limit FTI efficacy.

A comparison of preclinical tests, performed in transgenic mouse models of breast cancer, with clinical tests, in breast cancer patients, indicates that FTIs are far more efficacious in the mouse models than in the patients. Why? Mouse studies argue that the ability of FTIs to kill cells is a key factor in their efficacy [10], a finding that would surprise few. FTI will induce growth inhibition in most human breast cancer cell lines [15], but with the exception of a few lines (e.g. MCF7 [9]), most are not very susceptible to apoptosis. Genetic studies in the mouse prove that the ability of FTI to induce apoptosis depends upon gain-of-function in RhoB. Thus, one logical strategy to define factors that dictate apoptotic susceptibility is to compare the genetic response of cells with different RhoB genotypes to FTI-induced apoptosis. In the last period, we used a gene microarray hybridization strategy to focus specifically on events that precede the execution of RhoB-dependent apoptosis (rather than on other aspects of the FTI response mediated by RhoB gain-of-function, such as growth inhibition. By this approach [14], genes that control cell adhesion and cell shape were represented prominently among upregulated targets of RhoB, as were genes that control signal

transduction, vesicle dynamics, transcription, and immunity. Genes that control cell cycle checkpoints and progression through S phase and mitosis were among the major downregulated targets of RhoB. In support of the concept of RhoB as a negative regulator of Ras signaling pathways, the most strongly downregulated gene scored was farnesyl pyrophosphate synthetase, the enzyme that produces the substrate used by FT to farnesylate Ras proteins. Gene clustering revealed modules for MAPK signaling, cell cycle progression, and immune response as proapoptotic targets of RhoB. Progress has been made in identifying a key function role for the pivotal cellular regulator, cyclin B1, which was identified in the gene chip screen.

We had focused on the relevance of cyclin B1 suppression because of observations from several laboratories that suggest RhoB can affect events in mitosis, including events that impact apoptotic susceptibilities [16]. This work was published recently [16a] in *Cancer Research* (a PDF reprint is provided with the present report).

Cyclin B1 downregulation was observed to occur specifically in cells that were fated to undergo apoptosis after FTI treatment, a process which requires RhoB. In the last period, we showed that enforcing cyclin B1 expression in susceptible cells did not affect susceptibility to FTI-induced growth inhibition, morphological alteration, or actin reorganization but yet rendered cells resistant to FTI-induced apoptosis. These findings highlighted a specific effect of cyclin B1 on the survival of transformed cells that is distinct from its effects on cell cycle, insofar as cyclin B1-overexpressing cells did not cycle faster than control cells. We showed that enforcing cyclin B1 in drug susceptible cells abolished their in vivo response to FTI in a tumor allograft assay. This result confirmed the notion that cyclin B1 downregulation by RhoB is critical to antitumor activity. Studies of how RhoB regulates cyclin B1 revealed two mechanisms of action, one involving transcriptional control and a second involving posttranslational control. Studies of the former mechanism showed that gain of RhoB elicited by FTI treatment was sufficient to suppress cyclin B1 transcription, as measured by a cyclin B1 promoter-luciferase reporter vector. RhoB-GG (the geranylgeranylated isoform elevated by FTI in cells) was sufficient to phenocopy transcriptional suppression by FTI+RhoB. These

effects were specific insofar as the closely related but FTI-independent RhoA protein elevated rather than suppressed cyclin B1 promoter activity. Effector mutant studies suggested that the ability of RhoB to interact with the Rho effector molecule kinectin was important for this activity. Studies of the posttranslation mechanism showed that RhoB mediated the ability of FTI to drive preferential accumulation of cyclin B1 in the cytosol of cells, only under conditions where cells were are destined to die (rather than be growth inhibited). In contrast, nuclear accumulation was prevented. This effect occurred rapidly, within 8 hr of FTI treatment, and it only occurred in cells if RhoB-GG was generated. Interestingly, cyclin B1 accumulated in the cytosol in a punctate pattern suggested vesicular association, perhaps associated with degradation since at later times steady-state levels of cyclin B1 decreased dramatically. This result is interesting in light of the idea that RhoB may regulate a 'trafficking-to-trash' pathway involving the multivesicle body and the lysosome. We are currently examining this process in more detail. As mentioned above, these important findings have now been published [16a] in *Cancer Research 64: 8389 (2004)* – noted in the Highlights feature of the issue.

In the last grant period, we reported evidence that the Bin1 gene is necessary for FTI to trigger apoptosis via RhoB. Bin1 is a tumor suppressor gene that has been previously linked to breast cancer suppression and transformation-selective apoptosis [17-19]. To further develop the hypothesis that Bin1 is a cancer suppression gene, we knocked out this gene in the mouse [20]. Notably, transformed cells that lack Bin1 were found to be resistant to FTI-induced apoptosis [13]. The findings of this study argued that the adapter proteins encoded by the Bin1 gene acted downstream or in parallel to RhoB in cell death signaling. Certain Bin1 splice isoforms have been shown to associate with endosomal vesicles and to influence trafficking processes, like RhoB. In the next grant period, we plan to determine whether Bin1 may also influence cyclin B1 transcription or cytosolic accumulation by FTI/RhoB.

The identification of roles for Bin1 and cyclin B1 suppression in apoptosis by FTI are significant to issues surrounding the efficacy of FTI in breast cancer, because cyclin

B1 is frequently overexpressed and Bin1 is frequently attenuated in human breast cancers [17]. Thus, these two events may limit susceptibility to FTI treatment in the clinic.

Tasks 2a and 2b to generate and test the *in vitro* FTI response of cell populations where cyclin B1 and Bin1 is manipulated is complete. Task 2c to examine the *in vivo* FTI response of tumor allografts is complete, although we would like to confirm this with cell lines that extend beyond the model used initially. No progress was made this period on Task 2d to examine the hypothesized correlation between FTI response and cyclin B1 or Bin1 status in malignant human cell lines. However, we hope to interest investigators at Johnson & Johnson, which is performing Phase II/III clinical testing of the FTI R115777, in examining the question whether there is a relationship between cyclin B1 and Bin1 status in FTI responders and non-responders in clinic (via immunohistological analysis of tumor biopsy sections).

Key Research Accomplishments

1. Demonstration that RhoB knockout mice can be bred to MMTV-c-myc oncomice, a well-established model of breast cancer.
2. Finding that loss of RhoB leads to increased stability of the c-Myc protein.
3. Demonstration that RhoB suppresses the transcription of the cyclin B1 gene and alters the subcellular localization of cyclin B1, events that are critical in cells destined to undergo FTI-induced apoptosis.

Reportable Outcomes

1. Generation of a novel mouse model of breast cancer (MMTV-myc;rhoB KO virgin mice exhibit tumor formation at 4-6 mos.)
2. Publication of a manuscript at *Cancer Research* describing work on cyclin B1 as a proapoptotic effector target for RhoB signaling (Kamasani, U., Huang, M., DuHadaway, J., Prochownik, E.V., Donover, P.S. and Prendergast, G.C. (2004). Cyclin B1 is a critical target of RhoB in the cell suicide program triggered by farnesyl transferase inhibition. Cancer Res., 64: 8389-8496.).

Conclusions

Work in this grant period revealed genetic interactions between Rho and Myc in breast cancer. These gene families have major roles in driving tumorigenesis. Specifically, we have observed that attenuation of RhoB expression (as occurs in many cancers including breast cancers) elevates the stability of the c-Myc protein and promotes the formation of c-Myc-driven breast tumors. This interface is novel, exciting, and significant for its implications in how FTIs may influence via RhoB both breast tumor growth and Myc expression. During this grant period, we also advanced work on how cyclin B1 suppression by RhoB mediates the ability of FTI to kill transformed cells. Specifically, we found that cyclin B1 suppression was critical for the ability of FTI to inhibit the growth of tumor cells. We also identified a posttranslational mechanism of cyclin B1 regulation by RhoB, which involves preferential accumulation of cyclin B1 in the cytosol of cells destined to undergo FTI-induced cell death. This mechanism has importance to basic understanding of the regulation of cyclin B1, a pivotal player in cells. The pattern of cytosolic accumulation observed suggests that RhoB may regulate a 'trafficking-to-trash' pathway. Future work will focus on investigating this process as well as on the ability of RhoB to influence c-myc-driven breast cancer, c-Myc protein stability, and FTI responsiveness of c-myc-driven breast tumors.

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Appendices

Original Research Reports (preprints)

Kamasani, U., Huang, M., DuHadaway, J., Prochownik, E.V., Donover, P.S. and Prendergast, G.C. (2004). Cyclin B1 is a critical target of RhoB in the cell suicide program triggered by farnesyl transferase inhibition. Cancer Res. 64, 8389-8396.

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Cyclin B1 Is a Critical Target of RhoB in the Cell Suicide Program Triggered by Farnesyl Transferase Inhibition

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ABSTRACT

Farnesyl transferase inhibitors (FTIs) have displayed limited efficacy in clinical trials, possibly because of their relatively limited cytotoxic effects against most human cancer cells. Therefore, efforts to leverage the clinical utility of FTIs may benefit from learning how these agents elicit p53-independent apoptosis in mouse models of cancer. Knockout mouse studies have established that gain of the geranylgeranylated isoform of the small GTPase RhoB is essential for FTI to trigger apoptosis. Here we demonstrate that Cyclin B1 is a crucial target for suppression by RhoB in this death program. Steady-state levels of Cyclin B1 and its associated kinase Cdk1 were suppressed in a RhoB-dependent manner in cells fated to undergo FTI-induced apoptosis. These events were not derivative of cell cycle arrest, because they did not occur in cells fated to undergo FTI-induced growth inhibition. Mechanistic investigations indicated that RhoB mediated transcriptional suppression but also accumulation of Cyclin B1 in the cytosol at early times after FTI treatment, at a time before the subsequent reduction in steady-state protein levels. Enforcing Cyclin B1 expression attenuated apoptosis but not growth inhibition triggered by FTI. Moreover, enforcing Cyclin B1 abolished FTI antitumor activity in graft assays. These findings suggest that Cyclin B1 suppression is a critical step in the mechanism by which FTI triggers apoptosis and robust antitumor efficacy. Our findings suggest that Cyclin B1 suppression may predict favorable clinical responses to FTI, based on cytotoxic susceptibility, and they suggest a rational strategy to address FTI nonresponders by coinhibition of Cdk1 activity.

INTRODUCTION

Cell suicide processes are thought to play an important role in limiting cancer progression and therapeutic response. Although great progress has been made in identifying the basic mechanisms of apoptosis, much less is known about transformation-specific mechanisms of apoptosis that may relate more directly to cancer pathophysiology. Insights in this area may increase understanding of the pathophysiological roots of cancer progression as well as identify better strategies to trigger cancer-selective cell deaths.

Farnesyltransferase inhibitors (FTIs) trigger a unique p53-independent apoptosis in transformed mouse and rat cells, *in vitro* and *in vivo*, although they have much less effect on the survival of most nontransformed cells (1, 2). FTIs were developed originally as a strategy to attack the farnesylation requirement of oncogenic *Ras* in human cancers. However, it has become clear that the response of transformed cells to FTIs is based to a significant extent on factors beyond Ras targeting (3, 4). For example, it has been demonstrated that the small GTPase RhoB is an essential player in FTI-induced apoptosis (5). RhoB is unusual among small GTPases in that it exists in two differently isoprenylated populations in cells that have a unique localization, and perhaps a unique function, in cells (6). RhoB responds to FTI treatment by a gain-of-function mechanism characterized by depletion of the farnesylated isoform of RhoB (RhoB-F) but elevation of the geranylgeranylated isoform of RhoB (RhoB-GG; refs. 7, 8). Notably, a gain of RhoB-GG is sufficient to mediate many major facets of the cellular response to FTI *in vitro* and *in vivo* (5, 8–10). In particular, a genetic proof of the essential role of RhoB-GG in FTI-induced apoptosis has been offered by knockout mouse studies (5). Reinforcing this line of work, other studies have shown that RhoB limits cancer development and that it is critical for the apoptotic response of transformed cells to genotoxic stress (11, 12).

Operation of this apoptotic program is widely blunted in human cancer cells, which most studies show are susceptible to growth inhibition, but not killing, by FTI. This apoptotic impotence may be relevant to clinical experience, which has not tended to recapitulate the dramatic efficacy produced by FTI in certain preclinical models, particularly in Ras transgenic models (13–15) in which tumor regressions elicited by FTI treatment are associated with induction of apoptosis (16). Because one would expect efficacy and cytotoxicity to be linked, learning how RhoB facilitates FTI-induced apoptosis in mouse models may suggest insights into the relative resistance of human cancer cells.

Recently, microarray studies identified Cyclin B1 as a major target for down-regulation by RhoB in transformed mouse cells fated to undergo FTI-induced apoptosis (17). This finding was interesting because of earlier evidence that FTI and RhoB influence G_2-M phase events (12, 18), including Ras-independent control of Cyclin B1/Cdk1 activity (19). We, therefore, tested the hypothesis that the suppression of Cyclin B1 by RhoB may be a critical factor in the ability of FTI to trigger apoptosis.

MATERIALS AND METHODS

Cell Culture. The generation and culture of mouse embryonic fibroblasts (MEFs) transformed by adenovirus E1A and mutant H-Ras has been described previously (5, 11). E1A+Ras-transformed MEF cell populations that are heterozygous or nullizygous for RhoB are termed ER +/– or ER –/– cells, respectively (5). Heterozygous cells, which exhibit similar biological properties to homozygous wild-type cells (5, 11), are matched to nullizygous cells to control for the presence of the neomycin resistance cassette used for *rhoB* gene replacement (11). The specific FTI inhibitor L-744,832 (13) was added to cell cultures to a final concentration of 10 μmol/L when indicated. In some experiments, the structurally distinct peptidomimetic FTI inhibitors B581 or FTI-277 were used at the same concentration. Generation and culture of the rat intestinal epithelial (RIE) cell line transformed by an oncogenic V12 mutant of K-Ras, termed RIE/K-ras, has been described previously (8). MMEC/myc is a c-*myc*-transformed mouse mammary epithelial cell line (myc83 cells) that was established from an autochthonous mammary gland tumor arising in a mouse mammary tumor virus (MMTV)-*c-myc* transgenic mouse (20, 21). MMEC/myc cells were cultured in Richter's Medium (IMEM) supplemented with 2.5% fetal calf serum (FCS; Hyclone, Logan, UT), 10 ng/mL human epidermal growth factor (Invitrogen, Carlsbad, CA), 5 μg/mL insulin (Life Technologies, Inc., Rockville, MD), and penicillin/streptomycin (Life Technologies, Inc.).

Ectopic expression of Cyclin B1 was achieved in ER +/– cells by liposome-

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mediated transfection of a full-length human *cyclin B1* cDNA vector that has been described previously (22). Briefly, cells were seeded at 5×10^5 cells per well in a 6-well dish and were transfected the next day with 3 μg of the cyclin B1 or empty pBabe(puro) vectors. After 48 hours, cells were passaged into 100-mm dishes, were treated the following day with 1 μg/mL puromycin, and were expanded into mass culture for analysis. Cell populations were screened by PCR to confirm stable integration of the *cyclin B1* vector and by Western analysis to confirm elevated expression of Cyclin B1 protein. To attenuate expression of endogenous Cyclin B1, ER –/–cells were transfected stably with an RNA interference (RNAi) vector modified to include a puromycin resistance cassette and the following hairpin *cyclin B1* targeting sequence defined as effective in suppressing levels of expression of Cyclin B1 protein in transient 293 cell transfection assays. Briefly, the primers CATGGAGAGCTCCATGAGGTATTTGGCCGAAGCTTGGGCTAAGTATCTTATGGAGCTCTCCATGCTGTTTTTT and GATCAAAAAACAGCATGGAGAGCTCCATAAGATACTTAGCCCAAGCTTCGGCCAAATACCTCATGGAGCTCTCCATGCG were kinased, annealed, and ligated into the *Bse*RI and *Bam*HI sites of pSHAG-puro, a derivative of pShag-1,[4] which includes a puromycin resistance cassette inserted at the *Eco*RV site. The RNAi vector pShag-puro-cycB1 targets the sequence GGCCAAATACCTCATGGAGCTCTCCATGC in mouse *cyclin B1* (bases 957 to 985) or rat cyclin B1 (bases 936 to 964) message.

Cell Morphology and Proliferation. To document cell morphology changes, we treated cells the day after passaging with 10 μmol/L FTI L-744,832 or DMSO vehicle and photographed the cells 24 or 48 hours later. Anchorage-dependent proliferation was determined by sulforhodamine B (SRB) assay in a 96-well format. Anchorage-independent proliferation was determined in soft agar culture (ER MEFs) or on polyHEMA-coated dishes (MMEC/myc cells) as described previously (9, 23).

Apoptosis. FTIs have previously been shown to induce apoptosis of Ras-transformed cells under conditions of deprival of serum growth factors or substratum attachment (5, 23–25). For serum deprival experiments, 5×10^5 ER MEFs or RIE/K-ras cells were seeded onto 60-mm dishes and were treated 16 hours later with FTI L-744,832 or dimethylsulfoxide vehicle in DMEM containing 0.1% fetal bovine serum. For adhesion deprival experiments, 1×10^6 MMEC/myc cells were seeded onto polyHEMA-coated dishes in media containing FTI L-744,832 or vehicle in fully supplemented media. After 24–72 hours, cells were harvested by trypsinization, were washed once with PBS, were fixed in 70% EtOH, and were analyzed by flow cytometry. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was performed in some experiments with a commercial kit according to the manufacturer's protocol (Roche Molecular Biology, Indianapolis, IN). TUNEL-positive cells in the population were quantitated by flow cytometry with a FACscan cell analyzer (Becton-Dickinson, San Jose, CA).

Tumorigenicity Assay. Cells were tested for tumorigenic potential in ~8-week-old nude mice (Charles River, Cambridge, MA). Mice were given injections subcutaneously on the upper thigh of different legs of the same animal with 10^6 cells suspended in 200 μL of DMEM. Palpable tumors appeared at the injection site within 1 week and visible nodules of >0.5 cm were apparent by 2 weeks. For FTI trials, mice were dosed once daily when the tumor reached ~50 to 100 mm^3 by intraperitoneal injection with FTI L-744.832 (40 mg/day) or 30% DMSO carrier in 0.2 mL total volume as described previously (13). Tumor volumes were calculated with caliper measurements and the formula, $(\text{width})^2 \times \text{length} \times 0.52$.

Western Analysis. Cells were harvested by washing once in PBS before lysis in 1× radioimmunoprecipitation (RIPA) buffer supplemented with 10 μL/mL Protease Inhibitor Sets II and III (Calbiochem, San Diego, CA). Cellular protein was quantitated by Bradford assay, and 25 μg of protein was fractionated by SDS-PAGE. Gels were analyzed by standard Western blotting methods with antibodies to Cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, cat. no. sc-245), Cdk1 [Cell Signaling, Beverly, MA; PhosphoPlus Cdc2 (Tyr15) Antibody kit], Bcl-xL (BD Transduction Laboratories, Los Angeles, CA; cat. no. 610211), and actin (Santa Cruz Biotechnology, cat. no. sc-1616). Detection of the primary antibody was carried out with a chemiluminescence system for the detection of murine antibodies (Pierce, Rockford, IL).

Cellular Immunofluorescence. Cells were seeded onto coverslips in 24-well dishes and were treated the next day for various times with 10 μmol/L FTI L-744,832 or dimethylsulfoxide carrier. To monitor actin, we treated cells for 48 hours with FTI before fixing and staining with fluorescein-phalloidin (Molecular Probes, Eugene, OR) as described previously (26). To monitor Cyclin B1, we seeded cells onto coverslips in 24-well dishes, and the next day, we replaced the medium with DMEM containing 10% or 0.1% fetal bovine serum (dictating growth-inhibitory or apoptotic response to FTI, respectively). On the following day, cells were treated for 8 hours with FTI or vehicle carrier (control) before fixation and processing for immunofluorescence. Briefly, cells were rinsed twice with PBS, fixed 10 minutes in 4% formaldehyde, and permeabilized 10 minutes with 0.2% Triton X-100 in PBS. After nonspecific sites were blocked by incubation for 10 minutes in 2% bovine serum albumin–0.2%Triton X-100 in PBS, cells were incubated for 1 hour in PBS containing 1 μg/mL anti-Cyclin B1 (GNS1, Santa Cruz Biotechnology). Bound antibody was detected by incubation 30 minutes with FITC-conjugated goat antimouse IgG1, as recommended by the vendor (1031-02, Southern Biotechnology Associates). After the final wash, cells were stained 5 minutes with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis MO) to visualize nuclei and were mounted on slides with Fluoromount G (0100-01; Southern Biotechnology Associates, Birmingham, AL) for examination by immunofluorescence microscopy.

Cyclin B1 Promoter Reporter Assays. ER –/–cells were seeded into a 6-well dish at 5×10^5 cells per well. On the following day, cells were transfected with 0.2 μg of SV-βGal (200 ng) plus 1 μg of pGL2-luciferase (Promega, Madison, WI) or the *cyclin B1* promoter-reporter pGL2-cycB1-luc (22) and 0, 0.5, 1, 1.5, or 1.8 μg of the HA epitope-tagged RhoB or RhoA vectors or the corresponding no-insert control vectors that have been described previously (7, 8, 27). The total quantity of DNA in each transfection was normalized to 3 μg with empty vector corresponding to the different RhoB vectors used (7–9, 27). Cells were harvested 48 hours after transfection and were processed with a commercial kit (Dual Light System, cat. no. BD100LP, Applied Biosystems, Foster City, CA) and a fluorescence luminometer (Analytical Luminescence Laboratory, San Diego, CA).

RESULTS

Suppression of Cyclin B1 by FTI Is Linked to Gain of RhoB-GG and Apoptotic Cell Fate. Cyclin B1 expression is restricted normally to G_2-M phase by a complex set of regulatory events (28). This tight control is profoundly disrupted during malignant development, as illustrated by aberrant expression patterns of Cyclin B1, including during G_1 phase in cancer cells (29). Given the central role of Cyclin B1 in cell cycle control and cell survival, and its profound disruption in transformed cells, one would expect the alteration of Cyclin B1 to mediate alterations in the physiology of transformed cells. This gene was identified previously as a target for suppression by RhoB in E1A+Ras-transformed cells that were fated to undergo FTI-induced apoptosis (17). This regulatory connection was selective to transformed cells, because it was not seen in normal cells. Therefore, we explored its potential significance in E1A+Ras-transformed cells that were heterozygous or homozygous null for rhoB (termed ER +/–or ER –/–cells), with the potent and specific FTI L-744,832 to induce growth inhibition or apoptosis under normal-culture conditions or serum-deprival conditions, respectively, as described previously (5).

FTI caused a rapid and specific reduction in steady-state levels of cyclin B1 in a manner that was associated with the induction of RhoB-GG and apoptotic fate (Fig. 1*A*). This response was specific insofar as Cyclin D1 and Cyclin A (which are required for transit through G_1-S phase, and S-phase/G_2 phases of the cell cycle, respectively) were not affected by FTI treatment. Furthermore, cyclin B1 was not suppressed under normal-culture conditions in which FTI treatment caused growth inhibition but not apoptosis (24, 25). This response was not compound-specific because the same pattern of cyclin B1 suppression was produced by the structurally distinct FTIs B581 and FTI-277 (Fig. 1*B*). Under the same conditions, FTI treatment also reduced steady-state expression of Cdk1, the cell cycle

[4] Internet address: http://www.cshl.org/public/SCIENCE/hannon.html.

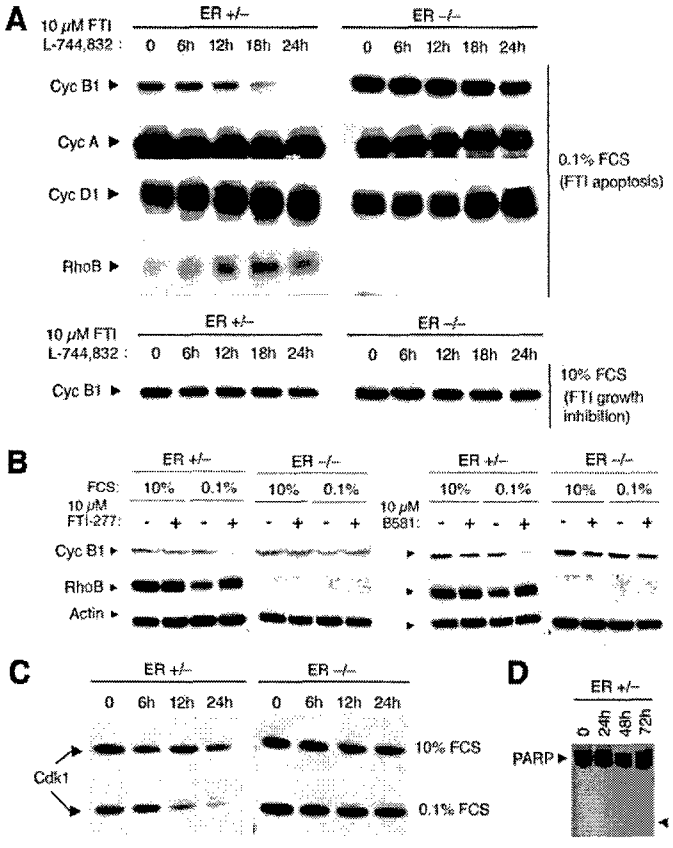

Fig. 1. Cyclin B1 suppression by FTI is associated with apoptotic fate and requires prior induction of RhoB-GG. *A*, steady-state levels of Cyclins B1 (*Cyc B1*), D1 (*Cyc D1*), and A1 (*Cyc A*) and RhoB in ER +/–and ER –/–MEFs after FTI treatment. Western analysis was performed with extracts prepared from cells that were cultured in serum-deprived conditions [0.1% FCS (*0.1% FCS*)], in which FTI will induce apoptosis, or from cells cultured in normal growth media (*10% FCS*), in which FTI will induce growth inhibition but not apoptosis (5). In *B*, Cyclin B1 responds similarly to structurally distinct inhibitors of farnesyltransferase. Western analysis was performed with extracts from cells under the same conditions as above except for treatment with the peptidomimetic inhibitors FTI-277 or B581. *C*, Cdk1 down-regulation accompanies Cyclin B1 suppression. Western analysis was performed as above with pan- or phospho-specific Cdk1 antibodies. In *D*, suppression of Cyclin B1 precedes execution of cell death. Western analysis of the caspase-3 indicator protein PARP is shown in extracts prepared from cells treated with FTI under serum-deprived conditions. PARP cleavage signals the execution phase of apoptosis, which occurs after Cyclin B1 suppression under the conditions of the experiment. (*h*, hour; *μM*, mmol/L.)

kinase that is specifically bound and activated by Cyclin B1 (Fig. 1*C*). The basis for Cdk1 loss was unclear, but it suggested a mechanism that focused on the Cyclin B1/Cdk1 complex. The specific reduction in Cyclin B1 and Cdk1 was not derivative of cell death, because Cyclin D1 and Cyclin A were not affected and because the kinetics of Cyclin B1/Cdk1 reduction preceded the kinetics of poly(ADP-ribose) polymerase (PARP) cleavage, an indicator of caspase-3 activation during the execution phase of apoptosis (Fig. 1*D*).

Cyclin B1 responded similarly to FTI in other cell systems in which neoplastic transformation was driven by FTI-insensitive oncoproteins. RIE/K-ras is an RIE rat intestinal epithelial cell line transformed by the activated K-Ras mutant K-RasG12V (8). FTI does not inactivate K-Ras, because this protein is geranylgeranylated in drug-treated cells (30). Nevertheless, despite the failure to block K-Ras prenylation, FTI strongly inhibits the proliferation of RIE/K-ras cells in serum-containing media, an effect that is associated with RhoB-GG induction and that can be phenocopied by ectopic expression RhoB-GG (8). In the absence of serum growth factors, we observed that FTI triggers apoptosis of RIE/K-ras cells, consistent with similar observations in other K-Ras-transformed cells (24). As before, apoptotic fate was associated with specific suppression of Cyclin B1 (Fig. 2*A*). A similar pattern was also observed in an epithelial cell system in which transformation was driven by c-Myc, which does not use Ras pathways to transform cells. MMEC/myc (also known as myc83) is a c-*myc*-transformed mouse mammary epithelial cell line that derived from *in vitro* establishment of an autochthonous MMTV-c-*myc* tumor (20, 21). MMEC/myc cells responded to FTI by growth inhibition unless cells were deprived of substratum adhesion (by culturing cells on the nonadherent substrate polyHEMA; 23). Cyclin B1 was suppressed by FTI only under conditions of adhesion deprival that led to cell death (Fig. 2*B*). Taken together, these observations extended the evidence that Cyclin B1 suppression was associated with apoptotic fate in settings in which Ras inhibition by FTI was irrelevant. In summary, suppression of Cyclin B1 by FTI was not a MEF-specific response, was not associated with the execution phase of cell death, and was not associated with a cell cycle arrest derivative of FTI-induced growth inhibition. We concluded that RhoB specifically mediated suppression of Cyclin B1 in cells fated to undergo FTI-induced cell death.

RhoB Mediates Inhibition of the Cyclin B1 Promoter by FTI. The identification of *cyclin B1* in gene microarray studies suggested that RhoB acted in part by affecting mRNA stability or promoter activity. RhoB was not observed to affect steady-state levels of Cyclin B1 protein driven from an exogenous promoter, which argues against the notion that mRNA stability was targeted (data not shown). In contrast, RhoB inhibited transcription from the cyclin B1 promoter as measured by a luciferase reporter plasmid (22). We studied this effect in ER –/–cells in which it was possible to analyze the properties of various transfected isoforms of ectopic RhoB on the *cyclin B1* promoter.

RhoB inhibited the activity of the *cyclin B1* promoter in a dose-dependent manner (Fig. 3*A*). This effect was not phenocopied by the related but functionally distinct RhoA protein, which activated the *cyclin B1* promoter (Fig. 3*B*). Consistent with the notion that RhoB mediated FTI action at this promoter, FTI had little effect unless RhoB was expressed ectopically (Fig. 3*C*). Moreover, an engineered RhoB-GG isoform (9) was sufficient to phenocopy the inhibition elicited by wild-type RhoB plus FTI (Fig. 3*C*). The requirement for an active RhoB molecule was illustrated by the inability of the CaaX mutant RhoB-C186C (27) to suppress the *cyclin B1* promoter. This observation reinforced the conclusion that the ability of FTI to inhibit Cyclin B1 expression was causally connected to the ability of FTI to elicit the geranylgeranylated isoform of RhoB in cells.

A panel of six effector domain mutations with known interaction properties (8) was used to address the question as to whether the pattern of effector interactions differed from those associated with growth inhibition by RhoB-GG [which map significantly to interactions with protein kinase C–related kinase (PRK) (8)]. No single mutant completely relieved the ability of RhoB-GG to inhibit the *cyclin B1* promoter, suggesting that multiple interactions were needed to mediate full inhibition. However, the activity pattern differed markedly from that seen for growth inhibition (8). In particular, the T37Y and E40T mutants suggested a role for kinectin, a Rho effector protein that binds the microtubule motor kinesin (Fig. 3*D*). T37Y is a mutant that abolishes interactions with all Rho effector molecules except kinesin (31), and this mutant was even more active than wild-type RhoB-GG. In contrast, E40T, which abolishes interactions with kinesin but not with other Rho effector molecules (31), relieved inhibition by RhoB-GG more strongly than any other mutant. Taken together, these results supported the interpretation that RhoB mediated the ability of FTI to suppress Cyclin B1 at a transcriptional level, by a mechanism(s) distinct from that used to cause growth inhibition.

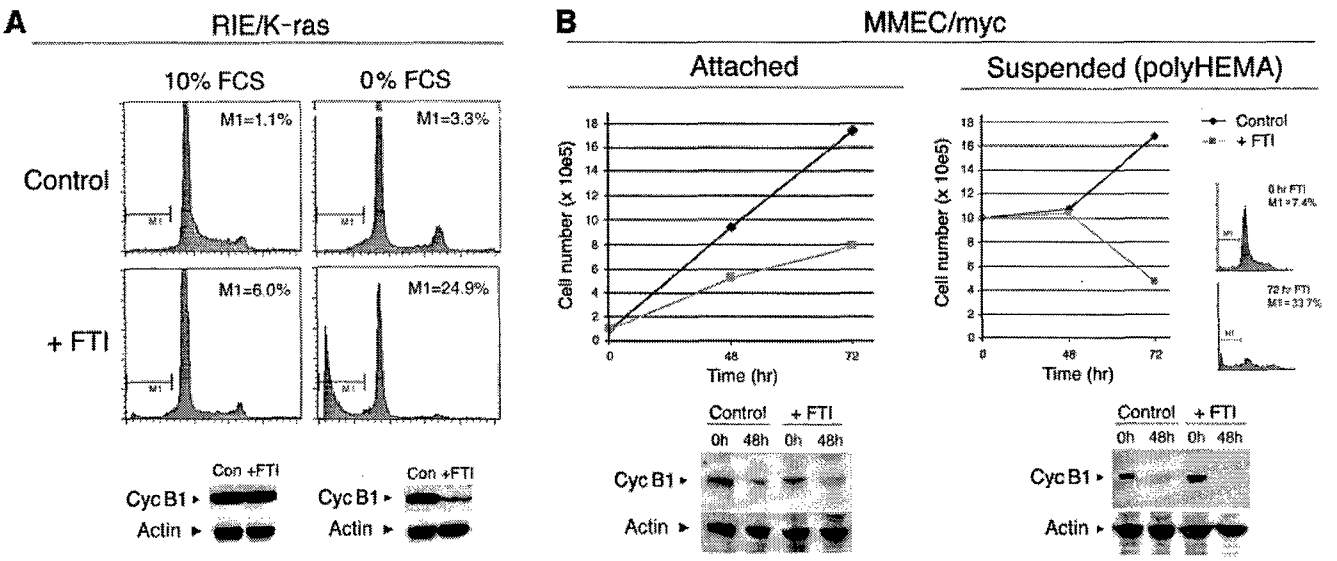

Fig. 2. Similar response of cyclin B1 to FTI in transformed epithelial cells. *A*, K-Ras-transformed rat intestinal epithelial cells (RIE/K-ras cells). Five $\times$ 10^5 cells were seeded overnight and were treated 16 hours with medium containing 10% FCS or 0% FCS before the addition of 10 μmol/L FTI L-744,832 or vehicle only. Cells were processed 24 hours later for flow cytometry (*top panels*) or Western analysis (*bottom panels*). *B*, c-*myc*-transformed mouse mammary epithelial cells (MMEC/myc cells). One $\times$ 10^6 cells were seeded onto standard or polyHEMA-coated tissue culture dishes in growth medium containing 10 μmol/L L-744,832 or vehicle only and then were processed at the times indicated for cell growth and flow cytometry (*top panels*) or for Western analysis (*bottom panels*). Viable cell number was determined by trypan blue exclusion with confirmation of cell death at 72 hours by flow cytometry, with sub-G_1 phase cells as a basis for quantitation. (*Con*, control only; *Cyc B1*, Cyclin B1; *h* and *hr*, hour; *M1*, gate M1.)

Cytosolic Accumulation of Cyclin B1 Precedes Suppression of Protein Levels and Apoptosis. RhoB has a specific physiologic function in signal trafficking as illustrated by its role in trafficking of the epidermal growth factor receptor (EGFR) and Akt (32, 33). Mistrafficking of these proteins that is elicited by FTI is associated with induction of RhoB-GG and altered protein turnover. Therefore, although Cyclin B1 had been highlighted by gene microarray analysis, we were interested in learning whether RhoB might influence its trafficking in transformed cells. As mentioned above, although Cyclin B1 is restricted to G_2-M phase in normal cells, its regulation is profoundly disrupted in neoplastic cells, in which inappropriate nuclear expression of Cyclin B1 can be observed broadly in the cell cycle including in G_1 phase (*e.g.*, see refs. 29, 34). E1A+Ras-transformed cells have been used widely as a cancer model, and consistent with the human cancer cell studies, we observed widespread constitutive nuclear expression of Cyclin B1 in unsynchronized cell populations (see below and Discussion).

To assess the effects of RhoB on the localization of Cyclin B1, we used indirect immunofluorescence to examine the status of Cyclin B1 in ER +/–and ER –/–cells treated 8 hours with FTI under conditions in which cell fate was directed to either growth inhibition or apoptosis (Fig. 4). This time point was early in the FTI response, within the time that RhoB-GG was induced but before any reduction occurred in the steady-state levels of Cyclin B1 (Fig. 1*A*) or induction of the effector phase of apoptosis (Fig. 1*D*). Two observations were made. First, we observed widespread and robust nuclear expression of Cyclin B1 throughout the unsynchronized ER cell population, regardless of rhoB genotype, arguing that E1A+Ras transformation profoundly disrupted the normal regulation of Cyclin B1. Second, we observed that FTI induced a punctate cytosolic accumulation of Cyclin B1 in cells fated to undergo apoptosis (Fig. 4). This event was not associated with growth inhibition, because Cyclin B1 did not relocalize in cells fated to undergo growth inhibition. Moreover, it depended on the ability of FTI to elicit RhoB-GG because relocalization did not occur in ER –/–cells in which RhoB-GG could not be induced. The change in Cyclin B1 localization could conceivably occur in one of two ways, either by imposing a block to nuclear import or by potentiating nuclear export. Nuclear export of Cyclin B1 is mediated by a CRM1-dependent mechanism (35). However, we found that the CRM1 inhibitor leptomycin B1 did not prevent relocalization of Cyclin B1 in ER +/–cells (data not shown), suggesting that cytosolic accumulation occurred as a result of a block to nuclear import. We concluded that, at early times after FTI treatment, RhoB impaired the nuclear accumulation of Cyclin B1 in cells that were fated to undergo apoptosis.

Enforcing Cyclin B1 Expression Limits RhoB-Dependent Apoptosis Triggered by FTI. We reasoned that if cyclin B1 suppression was essential for FTI-induced apoptosis, then overexpressing cyclin B1 to combat its down-regulation might reduce apoptotic susceptibility of ER +/–cells. Conversely, we reasoned that reducing cyclin B1 expression would elevate the sensitivity of ER –/–cells to FTI-induced apoptosis. To test these predictions, we compared the FTI response in ER +/–cells in which Cyclin B1 was augmented by ectopic expression, and in ER –/–cells in which Cyclin B1 levels were reduced by an RNAi strategy. As a control for apoptosis suppression, we overexpressed the antiapoptotic protein Bcl-xL in ER +/–cells.

Using a human *cyclin B1* vector (22), we achieved a several-fold increase in steady-state levels of Cyclin B1 protein in ER +/–cells (Fig. 5). FTI treatment of these cells (ER +/–cycB1 cells) still led to a reduction in steady-state levels of Cyclin B1, but only to the level of expression that was characteristic of untreated vector control cells (ER +/–vect cells). This observation was consistent with evidence that RhoB affected Cyclin B1 at more than at a transcriptional level (as suggested by the microarray analysis). From a functional standpoint, the ER +/–cycB1 cells were acceptable to test our hypothesis, because the steady-state level of Cyclin B1 in FTI-treated cells persisted to levels that were similar to those found in untreated ER +/– vector cells. An effect of exogenous Cyclin B1 was also reflected on steady-state levels of Cdk1 and phosphorylated (active) Cdk1, which were elevated in ER +/–cycB1 cells toward the levels of untreated control cells (Fig. 5*A*). We noted that Bcl-xL also elevated the level of endogenous cyclin B1 and Cdk1, hinting at some level of cross-talk in this system that was mechanistically undefined.

Overexpressed Cyclin B1 specifically inhibited the apoptotic response of ER +/–cells to FTI treatment. Under normal culture con-

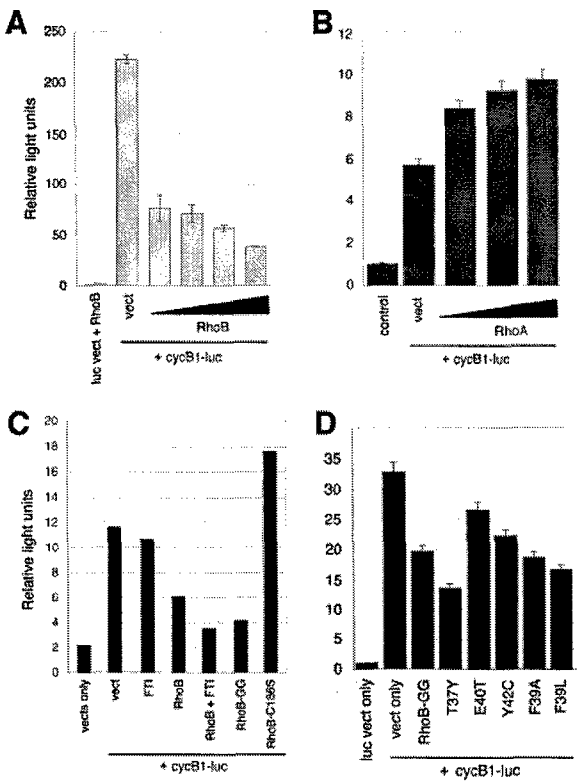

Fig. 3. Role of RhoB in suppression of the cyclin B1 promoter by FTI. ER –/–cells were transfected with an empty luciferase reporter plasmid (luc-vect), no-insert (vect), or Rho vectors as indicated in the figure panels and a human cyclin B1 promoter–luciferase reporter (*cycB1-luc*) at 1:1 to 1:5 (w/w) ratios to the Rho vector. All cells were cultured in low serum conditions for 24 hours before normalized luciferase activity was determined in cell extracts 48 hours after transfection. *Error bars*, the SD of the data. In *A*, RhoB inhibits the activity of the cyclin B1 promoter. In *B*, inhibitory effect of RhoB is not phenocopied by RhoA, which activates the cyclin B1 promoter. In *C*, RhoB-GG phenocopies the ability of RhoB + FTI to inhibit the cyclin B1 promoter. RhoB vectors were transfected at a 5:1 ratio to the cyclin B1 luciferase reporter plasmid in this experiment. The mutant RhoB-C186S is unprenylated and inactive because of loss of the cysteine in the CaaX prenylation motif (7). In *D*, effector domain mutations in RhoB-GG identify a correlation between cyclin B1 promoter inhibition and interaction with the Rho-binding protein kinectin (see Results). RhoB vectors were transfected at a 5:1 ratio to the cyclin B1 luciferase reporter plasmid in the experiment.

ditions, ER +/–cells respond to FTI by undergoing morphologic reversion, stress fiber formation, and growth inhibition (5); and enforced cyclin B1 expression did not alter the pattern of these responses. FTI-induced actin stress fiber formation was not affected (data not shown). RhoB-GG is sufficient and necessary to mediate this response (5), so the ability of FTI to induce stress fibers in ER +/–cycB1 cells confirmed that enforced Cyclin B1 did not interfere with the induction of a functionally competent RhoB-GG molecule. Similarly, neither enforced cyclin B1 nor Bcl-xL blocked the ability of FTI to cause growth inhibition under anchorage-independent conditions (Fig. 5*B*). In contrast, enforced cyclin B1 and Bcl-xL each inhibited the apoptotic response of ER +/–cells to FTI treatment that was manifested under serum-deprived conditions, as documented by TUNEL assay and flow cytometry (Fig. 5*C*). This observation suggested that Cyclin B1 responded downstream of RhoB in the pro-apoptotic mechanism elicited by FTI. In the converse experiment, we found that small interfering RNA (siRNA)-mediated blockade of cyclin B1 expression in ER –/–cells was not tolerated in stably transfected cell populations. Two cell populations that stably integrated a pShag-1-derived cyclin B1 RNAi vector exhibited reduced cyclin B1 expression and increased FTI sensitivity, relative to control cells, but these cell populations proliferated poorly and were unstable (data not shown). This result was unsurprising, given the requirement of cyclin B1 for cell survival (36); and the instability of cell populations expressing the siRNA reinforced the expectation that abolishing Cyclin B1 would be highly deleterious to cell survival. In summary, we concluded that Cyclin B1 suppression was essential for the RhoB-mediated program of apoptosis triggered by FTI.

Cyclin B1 Overexpression Blunts the Antitumor Efficacy of Farnesyl Transferase Inhibition. Apoptosis plays a major role in the ability of FTI to block tumor growth by ER cells (5); therefore, one might predict ER +/–cycB1 cells to exhibit FTI resistance *in vivo*. To examine this prediction, we compared the FTI response of cells grown as tumor grafts in immunodeficient nude mice. ER +/–vect or ER +/–cycB1 cells (10^7) were injected into the opposite thighs of the same animal to control for nonspecific environmental effects. On the basis of the marked response of control ER +/–cells to FTI in previous studies (5), five mice in each group were treated in this manner. Palpable tumors formed in each animal within several days of injection. One week after the graft was initiated, mice were assigned randomly to control or drug treatment groups, the latter of which was dosed once daily for 14 days by intraperitoneal injection with 40 mg/kg L-744,832 as described previously (5, 13). Control mice were given vehicle carrier only. Tumor volumes were calculated at various times during the experiment from caliper measurements as described previously (5). ER +/–cycB1 cells grew markedly more slowly in nude mice than did ER +/–vect cells (Fig. 6*A*), which exhibited growth kinetics similar to parental ER +/–cells (5). The effect of ectopic Cyclin B1 was unexpected given the lack of any discernable effect on *in vitro* proliferation. Nevertheless, in contrast to ER +/–vect tumors, which were strongly inhibited by drug treatment, the ER +/–cycB1 tumors were completely resistant to FTI (Fig. 6*A*). The germane effect of Cyclin B1 on the *in vivo* response to FTI treatment was apparent when the data were internally normalized to control for the growth effect of Cyclin B1 (Fig. 6*B*). We concluded that the suppression of Cyclin B1 mediated by RhoB was critical for FTI efficacy in this system.

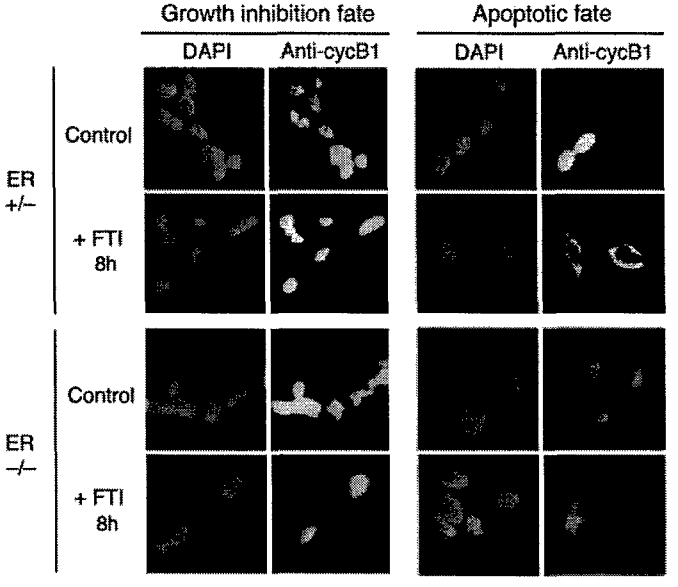

Fig. 4. Role of RhoB in mediating cytosolic accumulation of Cyclin B1 at early times after FTI treatment. ER +/–and ER –/–cells were seeded overnight onto coverslips and then fed with medium containing 10% or 0.1% fetal bovine serum (growth-inhibitory or apoptotic fate, respectively, after FTI treatment). On the following day, cells were treated 8 hours (*8h*) with 10 μmol/L FTI L-744,832 (*+FTI*) or vehicle (*Control*), were fixed and permeabilized, and were processed for indirect immunofluorescence with an anti-Cyclin B1 antibody (*Anti-cycB1*). DAPI costaining was used to visualize nuclei.

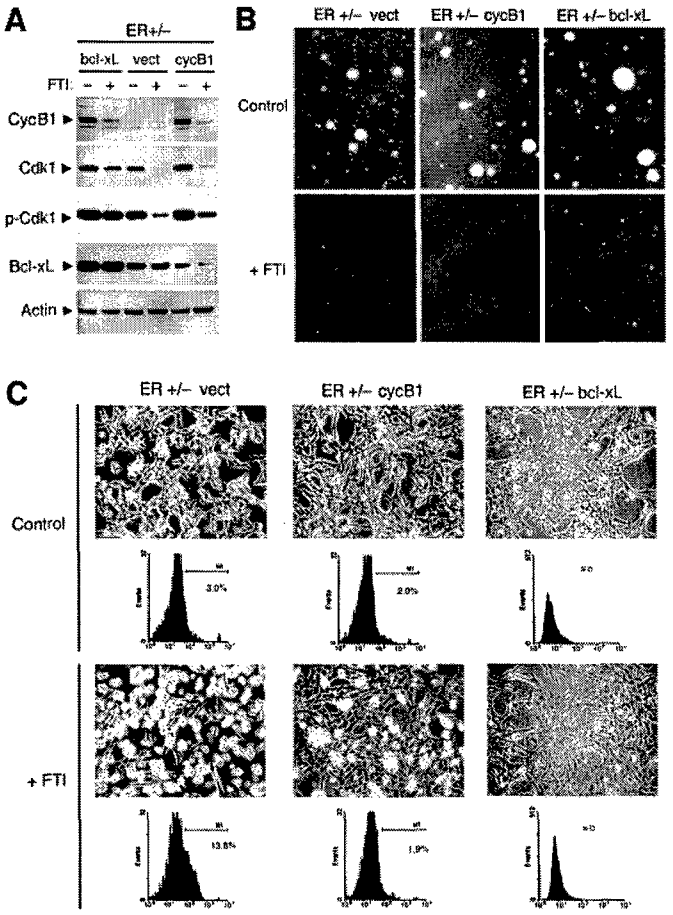

Fig. 5. Enforcing Cyclin B1 blocks apoptosis but not growth inhibition by FTI. *A*, transgene expression in ER +/−cell populations. Cells expressing ectopic Cyclin B1 (*cycB1*) or Bcl-xL (*bcl-xL*) or that contained only vector sequences (*vect*) were cultured 18 hours in medium containing 0.1% fetal bovine serum before 18-hour treatment with FTI (+) or vehicle only (−). Cell extracts were prepared and examined by Western analysis with antibodies to Cyclin B1 (*CycB1*), Cdk1, phospho-Cdk1 (*p-Cdk1*), Bcl-xL, and actin. *B*, lack of effect on anchorage-independent growth by FTI. Cells (10^4) were seeded in soft agar culture containing vehicle (*Control*) or FTI (+*FTI*), and colonies were photographed 2 weeks later. *C*, inhibition of apoptosis triggered by FTI. After seeding in normal growth medium, cells were fed with medium containing 0.1% fetal bovine serum and, 18 hours later, were treated an additional 24 hours with FTI (+*FTI*) or vehicle (*Control*) before photomicrography and processing for TUNEL reaction and flow cytometry. (*N.D.*, not determined.) M1 gate quantifies apoptosis by TUNEL.

DISCUSSION

This study identifies Cyclin B1 as a critical proapoptotic target of RhoB in the response of transformed cells to FTI treatment. Cyclin B1 was not affected under conditions of growth inhibition but was linked specifically to apoptotic induction. The earliest changes exhibited by Cyclin B1 occurred within 8 hours of FTI treatment, after induction of RhoB-GG but long before induction of cell death. The notion that Cyclin B1 is required for cell survival is well supported by genetic studies in the mouse (36). By elucidating a specific and essential component of the apoptotic program triggered by FTI, this study addresses the question of how cytotoxic *versus* cytostatic responses to FTI may be determined. Efforts to address this question are important, because they may promote efforts to learn why FTI is not cytotoxic or efficacious to human cancers and to learn how to leverage the utility of these well-tolerated agents in the clinic. Our results argue that suppressing Cyclin B1 is critical to antitumor activity, in support of other evidence that apoptosis is critical to *in vivo* antitumor efficacy (5, 14, 16). In the one study that has reported FTI effects on cyclin B1 in human cancer cells, it was found that growth inhibition was associated with high Cyclin B1/Cdk1 activity, which is consistent with our observations (19). By establishing a causal link between Cyclin B1 suppression and apoptosis, this study advances understanding of how FTI triggers transformation-selective apoptosis via RhoB-GG. The connection between FTI and Cyclin B1 suppression is important because it may help uncover potential defects in a pathway that is responsible for attenuating the cytotoxicity and efficacy of FTIs in human cancers.

RhoB localizes to plasma and to vesicular and nuclear membranes, and it has a physiologic function in controlling intracellular trafficking processes (6, 33, 37–39). Knockout mouse studies show that RhoB is dispensable for murine development but that it has a critical role in stress-signaling processes and cancer suppression (2). Existing members of the RhoB "traffickome" include the EGFR and the survival kinase Akt, two important regulators of neoplastic cell growth and survival (32, 33). The findings of this study suggest that trafficking of Cyclin B1 may also be subjected to control by RhoB under certain stress conditions, including those present in transformed cells. Thus, RhoB may influence susceptibility to proapoptotic stimuli by influencing how signaling molecules are trafficked under stressful conditions (*e.g.*, "trafficking to survive" *versus* "trafficking to trash"). In support of the concept that signal trafficking may influence apoptotic susceptibility, another study has defined an essential role for *Bin1/Amphiphysin2* [a BAR adapter-encoding gene implicated in vesicle trafficking processes and cancer suppression (40–45)] in FTI-induced apoptosis (46). The present work develops the model that altered-signal trafficking can alter apoptotic susceptibility to FTI. If selection against proapoptotic trafficking processes occurs during malignant

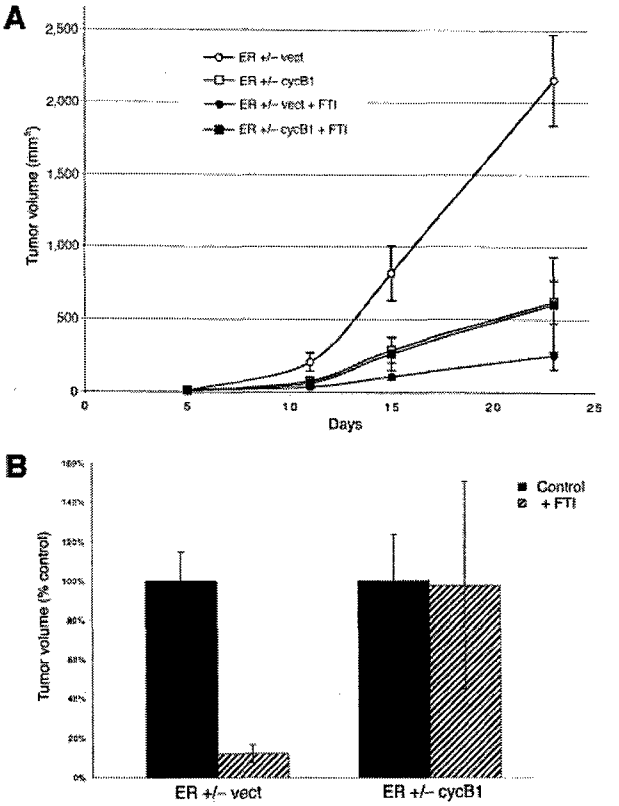

Fig. 6. Enforcing Cyclin B1 abolishes FTI antitumor activity. *A*, tumor growth curve. Cells (10^7) were injected subcutaneously into nude mice, and tumor volume was determined by caliper measurements at times afterward ($n = 5$). *B*, relative effect of FTI on tumor growth at 2 weeks. The data were internally normalized to each cell line to control for the apparent growth effect of Cyclin B1 (*cycB1*) on tumor growth. *Error bars*, SD of the data. (*vect*, vector.)

development, as it does for proapoptotic signaling processes, then such events may be expected to modify the resistance of human cancer cells to FTI-induced apoptosis.

Gaps in understanding Cyclin B1 control in cancer cells complicate the interpretation of our findings. In particular, emerging evidence suggests that mechanisms that normally restrict nuclear accumulation of Cyclin B1 to G_2-M phase are profoundly disrupted in human cancer cells (29, 34). Nuclear accumulation of Cyclin B1 documented in G_1 phase in cancer cells (29) would be expected to elicit premature mitosis and cell death in normal cells, suggesting radical differences in not only the regulation but also the function of Cyclin B1 in cancer cells. E1A+Ras-transformed cells have been used widely to model cancer; and, consistent with human cell studies, we observed widespread constitutive nuclear expression of Cyclin B1 in our E1A/Ras-transformed murine fibroblasts. The significance and basis of these observations are unclear at present, but they highlight the gaps in knowledge that persist about Cyclin B1 control, which is not generally well elucidated, even in normal cells. Although a possible role for cyclin B1 can be entertained in mediating RhoB-dependent cell deaths that occur in noncancer settings, it remains the case that one cannot fully interpret the present findings without gaining greater understanding of the basis and significance of the aberrant control of Cyclin B1 in neoplastic cells.

Despite this situation, it is possible to illustrate how our findings advance FTI studies. First, the Cyclin B1 response can be exploited further to elucidate the mechanistic linkage between RhoB and FTI-induced apoptosis. For example, a potential effector role for kinectin dovetails with the accepted signal-trafficking function of RhoB, because kinectin acts to anchor the microtubule motor protein kinesin to membranes and to facilitate vesicle movement (47). Kinectin is dispensable for normal development and physiology in the mouse, but its role in stress processes or cancer has not been explored (48). If kinectin is essential for Cyclin B1 control by RhoB, then defects in kinectin structure or expression in cancer may attenuate the ability of RhoB-GG to mediate apoptosis. If such defects are selected during tumor progression, they may inherently compromise FTI cytotoxicity. In summary, although kinectin has not been validated as a relevant effector molecule, the discussion above illustrates how learning about effectors can yield new clues to cancer pathophysiology and mechanisms of FTI cytotoxicity and resistance. Another use of the findings is the potential for Cyclin B1 to predict favorable *versus* unfavorable FTI responses in the clinic based on cytotoxic susceptibility. To date, clinical experience suggests that breast cancers and leukemias are among the most likely to respond favorably to FTI (49). Cyclin B1 has been reported to be profoundly dysregulated in these cancers, perhaps making them more susceptible to FTI cytotoxicity (29, 34). Given that overexpressed Cyclin B1 can limit FTI-induced apoptosis, an additional implication is that FTI cytotoxicity and clinical response might be enhanced by combination with a Cdk1 inhibitor. Indeed, generalized Cdk inhibitors have been reported to cooperate with FTI to trigger cell death in human cancer cells (50). Additional studies of Cyclin B1 in the FTI response of human cancer cells would seem warranted, given its potential to serve as a marker to help triage patients, predict responses, and address nonresponders in clinic.

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