RhoB is a ubiquitous member of the Rho family of isoprenylated small GTPases that control cytoskeletal actin organization in cells. RhoB is closely related to RhoA, its better-studied relative. However, RhoB differs in a number of aspects that indicate it has unique cellular functions. First, RhoB is located in early endosome and nuclear membranes (1, 14, 15, 20, 26). Second, RhoB appears to have a specialized role in intracellular trafficking of cytokine receptors such as the epidermal growth factor receptor (9). Third, unlike most small GTPases, RhoB is short lived and is part of the immediate early genetic response to epidermal growth factor, transforming growth factor β (TGF-β), Src activation, or genotoxic stress (5–8, 11, 12). Lastly, RhoB is a crucial target for alteration by farnesyltransferase inhibitors, which selectively inhibit the proliferation and survival of transformed cells (3, 4, 14, 17).

To investigate the physiological functions of RhoB, we analyzed mice in which the rhoB gene was targeted for homologous deletion. Loss of RhoB produced a minimal phenotype that was not associated with any apparent effects on development, fertility, or wound healing. However, cellular analyses revealed roles for RhoB in motility and proliferation responses that were associated with stress conditions, including those elicited by in vitro culture and neoplastic transformation. Interestingly, RhoB loss in mice was associated with an increased susceptibility to chemical carcinogenesis, and transformed cells lacking RhoB were more efficient at forming intraperitoneal tumors. Our findings suggest that RhoB has a negative regulatory or modifier function in neoplastic cells.

Materials and Methods

Construction of rhoB nulligous mice. The gene-targeting plasmid used to replace the single exon encoding the murine RhoB protein by homologous recombination was generated as follows. One-kilobase and 5.4-kb EcoRI genomic fragments from the murine rhoB locus were cloned into pBluescript SK (+) separately, generating the plasmids pR1 and pR5; pR1 was digested with Apal and BamHI, blunt end filled, and ligated to a 4.8-kb XhoI-XbaI fragment containing an internal ribosome entry site (IRES)-tau-lacZ gene, generating pR12; pR5 was digested with BamHI and ligated to a 1.8-kb BamHI fragment containing pGKneo, generating pR51. The final target plasmid was generated by ligating a blunt-ended SalI-SpeI fragment from pR120 into the NotI site of pR51. Standard methods were used to electroporate Sv129 embryonic stem (ES) cells with a linearized preparation of the targeting construct produced by SalI digestion. Ten ES clones from 288 clones screened had undergone the desired homologous recombination event to replace one rhoB allele. Mouse C57Bl/6 blastocysts were injected by standard methods with three different targeted ES cell clones (137, E, F). Chimeric mice exhibiting germ line transfer of the targeted allele were obtained from all three clones. The genotype of mice and cultured embryo fibroblasts was confirmed by PCR and Southern blot analyses as described previously (17).

Cell culture. Mouse embryo fibroblasts (MEFs) were generated as follows. The heads, limbs, and internal organs were removed from embryos at E14.5 or E16.5. The carcasses were minced in Dulbecco modified Eagle medium (DMEM; Life Technologies) and individually trypsinized for 20 min at 37°C. Fetal bovine serum was added to stop the trypsinization, and the cell suspension was seeded into 25-cm² flasks containing DMEM and 10% fetal bovine serum. Cells were maintained in the same media containing 10 U of penicillin-streptomycin per ml. MEF cell lines generated by cotransformation with the human oncogenic H-Ras vector pT22 and the adenovirus E1A vector p1A/neo have been described previously (17).

To monitor cell motility, attachment, and spreading, cells were seeded into dishes coated overnight at 4°C with 10 μg of fibronectin (Sigma) per ml dissolved in phosphate-buffered saline (PBS). For motility, a section of a confluent cell monolayer was cleared with a pipette tip, and the ability of cells to migrate into the cleared section in the presence of the cell division inhibitor mitomycin C was monitored. For spreading, 10⁶ cells were seeded in six-well dishes, and spread cells were photographed and counted after various periods. For attachment, a
fibronectin-coated 96-well dish was washed with PBS and incubated for 30 min at 37°C with 1 mg of bovine serum albumin per ml dissolved in PBS. Cells (10^4) were seeded into each well of the dish. At various times after seeding, the plate was washed with PBS and cells remaining attached were fixed for 20 min with 100 μl of 2% paraformaldehyde per well. Fixed cells were washed once with PBS and then stained for 20 min with 0.025% crystal violet in PBS. Stained cells were washed gently with water, and the absorbance at 540 nm was determined for each well using a microplate reader.

Growth curve determinations and colony formation assays performed in soft agar culture were performed essentially as described previously (3). For TGF-β response, 8,000 cells were seeded per well in quadruplicate in 96-well dishes and treated for 4 days with the indicated concentrations of TGF-β (Sigma). After the incubation period, 10 μl of 0.5-mg/ml (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) was added to each well. Following a 4-h incubation, cells were solubilized by overnight treatment at 37°C in 10% sodium dodecyl sulfate−0.01 M HCl. The absorbance of each well was determined at dual wavelengths of 570 and 650 nm using a microplate reader.

**Actin staining.** Cells were processed for staining with fluorescein-phalloidin (Molecular Probes) and indirect immunofluorescence microscopy as described previously (22).

**Western blot analysis.** Cells were washed in cold PBS and lysed in 1% NP-40 lysis buffer. Cellular protein was quantitated by Bradford assay, and 50 μg of cellular protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were analyzed by standard Western blotting methods using the β1 integrin antibody (catalog no. 141720; Transduction Laboratories). Detection of the primary antibody was carried out using a chemiluminescence system for the detection of murine antibody (Amersham).

**Tumor formation.** Twenty adult +/- and +/- mice were shaved, and the dorsal epidermis was treated with a single dose of 7,12-dimethylbenz[a]anthracene (DMBA) followed by twice weekly application of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), essentially as described previously (10). The number and size of papillomas on each mouse were recorded twice weekly. Tumor formation by E1A-plus-Ras-transformed cells was assessed by implantation of 2 × 10^3 cells into the peritoneal cavities of female Sv129 syngeneic mice. Mice were sacrificed 2 weeks later, and tumor nodules in the peritoneum were counted.

**RESULTS**

**rhoB deletion does not compromise mouse development or fertility.** Standard homologous recombination technology was used to delete the single exon encoding RhoB in mice. The targeting strategy is shown in Fig. 1. The targeting plasmid replaced an Apal-BamHI mouse genomic fragment containing the entire rhoB gene with IRES-tau-lacZ and pGKneo sequences. Three ES cell lines exhibiting the desired gene targeting event as indicated by Southern analysis were used to generate chimeric mice, all of which showed germ line transmission of the targeted allele (Fig. 1). Homozygous null animals were produced at Mendelian ratios, and no defects in development or fertility were apparent (data not shown), suggesting that RhoB was dispensable for these processes.

**Primary +/- MEFs exhibit a defect in motility on fibronectin.** Rho proteins regulate actin structures that are important for the adhesion, spreading, and motility of cells. Therefore, we compared these parameters in primary +/- and +/- MEFs. Attachment was assessed by comparing the number of +/- and +/- MEFs remaining at times after seeding equal numbers of cells into fibronectin-coated culture dishes and washing multiple times with PBS. Spreading was assessed by comparing the rates at which +/- and +/- MEFs flattened on dishes after seeding. No differences were noted in these tests (data not shown). Motility was assessed by comparing the rate at which +/- or +/- MEFs moved into a cleared section of a confluent monolayer in the presence of the cell division inhibitor mitomycin C. In this assay, a defect in motility of +/- MEFs was apparent. Within 18 h, +/- cells had migrated into the cleared section of the fibronectin-coated dish, whereas there was comparatively little movement of +/- cells (Fig. 2A). Cell motility is important during development, but as indicated above, we did not detect any phenotypic abnormalities in +/- mice. In addition, no motility defects were apparent during the healing of various types of skin wounds generated in +/- mice (data not shown). Thus, we interpreted the in vitro defect to be conditional on the presence of cell stresses generated by in vitro culture. In support of the defect observed, we noted an altered gel mobility of the β1 integrin fibronectin receptor subunit in +/- MEFs (Fig. 2B). This observation supported the likelihood of some disruption in substratum adhesion that could impact motility.

**Neoplastically transformed +/- MEFs exhibit altered adhesion and spreading.** It has previously been demonstrated that RhoB is a crucial target for alteration by farnesyltransferase inhibitors (FTIs), a class of experimental antineoplastic drugs. One of the most interesting aspects of FTIs is that they dramatically affect transformed cells but have little effect on normal cell physiology. It was reasoned that RhoB may have a similarly peculiar association with transformed cell physiology, perhaps due to stresses engendered by transformation. To explore this possibility, a comparison was made of the adhesive and motile properties of MEFs that were transformed by adenovirus E1A plus mutant Ras (17). For clarity, and to distinguish these cells from normal MEFs, we have used the designation ER for E1A-plus-Ras-transformed MEFs below.

In contrast to normal MEFs, we found that ER^-/- cells displayed a significant reduction in the rate of substratum attachment and spreading. After seeding equal numbers of cells on dishes coated with fibronectin, unattached cells were removed at various times by washing with PBS. Cells that were attached were stained with crystal violet and their relative number was quantitated by absorbance at 540 nm on a plate reader. Attachment of ER^-/- cells was markedly less efficient than that of ER^-/- cells 60 min after seeding on fibronectin (Fig. 3A). This phenotype was found to be associated with a reduction in the rate of cell spreading on fibronectin (Fig. 3B).
In time course experiments, ER \(-/\) cells spread significantly more slowly than ER \(+/-\) cells after seeding on fibronectin (Fig. 3C). The differences in spreading that were apparent at earlier times disappeared at later times, when ER \(-/\) cells were spread in a proportion similar to that of ER \(+/-\) cells (data not shown). Taken together, these findings suggested that RhoB affected cell adhesion capacity in some manner that was conditional on transformation or on stresses that were elicited by transformation.

**Altered response of transformed \(-/-\) MEFs to serum and to TGF-\(\beta\)**. RhoB has been reported to attenuate responses to TGF-\(\beta\) (5). However, we did not detect differences in the response of \(-/-\) primary MEFs to treatment with this growth factor (data not shown). Given the other differences in ER \(-/\) cells, we reasoned that transformation might uncover differences in the response to TGF-\(\beta\). It has been shown that under suboptimal serum conditions Ras-transformed cells respond to TGF-\(\beta\) by forming increased numbers of actin stress fibers through a process that is Rho dependent (21). In growth media, ER \(-/-\) cells exhibited no significant differences. In contrast, in the absence of serum growth factors ER \(+/-\) cells retained a limited stress fiber network, whereas ER \(-/-\) cells were essentially devoid of stress fibers (Fig. 4A). Under these conditions, both ER \(+/-\) and ER \(-/-\) cells responded to TGF-\(\beta\) by increased stress fiber formation. However, ER \(-/-\) cells were more sensitive to TGF-\(\beta\) and exhibited a robust response at lower concentrations of the growth factor (Fig. 4A). These observations suggested that RhoB was dispensable but that it modified the efficiency of TGF-\(\beta\) action.

These observations were extended by evidence that ER \(-/-\) cells also varied in their proliferative response to TGF-\(\beta\). In monolayer culture, ER \(-/-\) cells responded more robustly to

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**FIG. 2.** Defective motility of \(-/-\) MEFs on fibronectin and altered gel mobility of the \(\beta_1\) integrin isoform. (A) Motility. The ability of MEFs in a confluent monolayer to migrate into a section of the dish cleared by a pipette tip was documented by photography after 18 h in the presence of the cell division inhibitor mitomycin C (10 \(\mu\)M). (B) Western analysis of \(\beta_1\) integrin. Extracts isolated from MEFs of the genotype indicated were analyzed by Western blotting using a \(\beta_1\) integrin antibody. Note the slight reduction in the gel mobility of the upper band in \(-/-\) MEFs.
TGF-β also suppressed proliferation, except that in this case ER +/− cells were more susceptible to inhibition than were ER −/− cells (Fig. 4C). Thus, it appeared that adhesion status was an important factor in determining how RhoB impacted proliferation. Taken together, these results indicated that RhoB modified the response to TGF-β in a manner that was conditional on transformation and adhesion parameters.

**rhoB deletion promotes tumor formation.** The ability of RhoB loss to influence proliferation and adhesion in transformed cells suggested that it might affect tumorigenesis. To investigate this possibility, we compared the susceptibilities of −/− mice to tumor formation by two different routes. First, we performed a classical carcinogenesis test by initiating skin tumors with a single application of DMBA followed by multiple applications of the tumor promoter TPA. In this model for tumor progression, H-Ras mutation is the major initiating event (2, 25, 27). Twenty +/− and −/− mice were tested, and the number of skin tumors on each mouse was determined over a period of 16 weeks (Fig. 5A). −/− mice were more susceptible to the formation of benign skin papillomas, which predominate in the model. −/− mice bore a greater number of tumors on average, but during the period monitored we did not note any significant difference in tumor size or in susceptibility to carcinoma formation.

As a second method to examine the effects of rhoB deletion on tumor formation, we compared the consequences of implanting ER −/− cells into the peritoneal cavity of syngeneic animals. In previous experiments, ER −/− cells were shown to be similarly efficient in forming subcutaneous tumors in immunocompromised scid mice (17). However, in experiments where ER cells were injected into the intraperitoneal cavity of syngeneic Sv129 mice, we observed a marked difference in the number of tumor nodules formed at necropsy 2 weeks after injection (Fig. 5B). We concluded that RhoB loss promoted tumor formation.

**DISCUSSION**

The findings of this study suggest that RhoB is a modifier of adhesion and growth factor signals that are associated with cellular stress, in particular stresses associated with neoplastic transformation. A defect in MEF motility was detected. This defect might relate to inappropriate integrin trafficking at some level. RhoB has been suggested to have a specialized function in intracellular receptor trafficking (9), and differences in β1 integrin mobility on gels suggestive of a difference in posttranslational modification were observed. We interpreted the defect in MEF motility to be conditional on stress, however, because (i) −/− mice developed in an apparently normal manner; (ii) −/− mice did not display any defects in wound healing, where motility defects would be expected to be manifested; and (iii) in vitro culture subjects MEFs to stress (for an example, see reference 19). In support of some role in motility, RhoB has been implicated in the delamination of neural crest cells during chick development (18). However, the mechanisms governing this role may have varied during evolution, insofar as stress appears to be a prerequisite for RhoB to influence cell motility in mouse cells.

Through studies of the antineoplastic properties of FTIs we have previously documented a specialized connection between

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**FIG. 3.** ER −/− cells display reduced attachment and spreading on fibronectin. (A) Attachment. ER cells were seeded into six-well dishes coated with fibronectin, and 60 min later the cells were washed three times with PBS. Cells remaining attached were stained with crystal violet and the optical density (OD) at 540 nm was determined to quantitate the relative cell numbers. (B) Spreading morphology. ER cells were photographed 60 min after seeding on fibronectin-coated dishes. (C) Spreading time course. The relative proportion of non-fragile (spread) ER cells was determined at various times after seeding on fibronectin-coated dishes.
RhoB function and neoplastic transformation. These studies indicated that FTIs act not by eliminating RhoB function but instead by elevating a geranylgeranylated RhoB isoform that negatively impacts the proliferation and survival of transformed cells (3, 4, 17). This gain-of-function mechanism is compatible with the findings of this study, which argues that RhoB has a negative regulatory or modifier function in transformed cells. FTIs may to a large degree mediate their anti-

**FIG. 4.** RhoB loss alters the cytoskeletal actin and proliferative responses to TGF-β. (A) Actin response. Cells were seeded overnight, and growth medium was replaced with DMEM containing no serum plus the indicated concentration of TGF-β. Cells were processed for F-actin staining with fluorescein-phalloidin 24 h later. (B) Proliferation in monolayer culture. The MTT assay was used to monitor cell proliferation. ER cells were deprived of serum for 24 h and then fed medium without serum (control) or containing 10% serum plus the indicated concentration of TGF-β. Cells were processed for the MTT assay 72 h later. Open bars, ER+/−; solid bars, ER−/−. (C) Proliferation in soft agar culture. Cells were seeded into soft agar culture to monitor anchorage-independent proliferation, and the number of colonies relative to the untreated control was determined 14 days later.
transforming effects by accentuating an intrinsic negative regulatory function of RhoB. Previous findings suggesting a positive role for Rho proteins in transformation were gained by the use of dominant inhibitory mutants of RhoA or RhoB (13, 23, 24). These mutants broadly block endogenous Rho functions by competing for a variety of Rho exchange factors. By specifically eliminating RhoB we have shown that this Rho protein has a negative role rather than a positive role in transformation. A tumor-prone phenotype was revealed by the increased propensity of rhoB null mice for DMBA-induced papilloma formation. The results were consistent with an effect on the kinetics of tumor initiation, but it would be premature to conclude that RhoB loss acts in this way without additional investigation. Ongoing “oncousome” crosses will allow this issue to be assessed and possible tumor suppressor or modifier roles for RhoB to be examined further.

Our findings confirm previous evidence that RhoB attenuates growth factor responses in established cells (5, 9). A focus on TGF-β was stimulated by observations linking RhoB to this context-dependent regulator of transformed cell growth. TGF-β stimulates actin stress fiber formation in Ras-transformed cells in a manner that is associated with upregulation of RhoB and RhoA and that is reversed by the generalized Rho inhibitor C3 transferase (21). Here we showed that although RhoB was dispensable for TGF-β-induced stress fiber formation in transformed cells, it sensitized the cells to this process, a result that argues for its participation in the TGF-β response. Similarly, while RhoB loss did not abolish the effects of TGF-β on transformed cell proliferation, it modified the response in an adhesion-dependent manner. While we did not identify the basis for these biological effects, preliminary results from gene hybridization experiments argue against the notion that there are differences in receptor levels in cells (N. Rane, unpublished observations). Taken together, these observations prompt further investigations of how RhoB may influence TGF-β signaling in cells, particularly in the context of transformation or other stress- or disease-associated states.

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