

## ***REERG* Is a Novel *ras*-related, Estrogen-regulated and Growth-inhibitory Gene in Breast Cancer\***

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Using microarray analysis, we identified a unique *ras* superfamily gene, termed *REERG* (*ras*-related and estrogen-regulated growth inhibitor), whose expression was decreased or lost in a significant percentage of primary human breast tumors that show a poor clinical prognosis. Importantly, high *REERG* expression correlated with expression of a set of genes that define a breast tumor subtype that is estrogen receptor-positive and associated with a slow rate of tumor cell proliferation and a favorable prognosis for these cancer patients. *REERG* mRNA expression was induced rapidly in MCF-7 cells stimulated by  $\beta$ -estradiol and repressed by tamoxifen treatment. Like *Ras*, *REERG* protein exhibited intrinsic GDP/GTP binding and GTP hydrolysis activity. Unlike *Ras* proteins, *REERG* lacks a known recognition signal for COOH-terminal prenylation and was localized primarily in the cytoplasm. Expression of *REERG* protein in MCF-7 breast carcinoma cells resulted in a significant inhibition of both anchorage-dependent and anchorage-independent growth *in vitro* and inhibited tumor formation in nude mice. These features of *REERG* are strikingly different from most *Ras* superfamily GTP-binding proteins and suggest that the loss of *REERG* expression may contribute to breast tumorigenesis.

Microarray analysis of primary human tumors has led to the identification of novel tumor subtypes that were not identified previously and to the discovery of new genes that may be involved in the disease process. When using hierarchical clustering to classify breast tumors based upon variations in gene expression patterns, ~4–5 distinct breast tumor subtypes were identified (1, 2). In addition, it has been shown that these tumor subtypes were predictive of disease outcome (2); in the extended study of Sørlie *et al.* (2), the breast tumor data was statistically analyzed to identify genes whose expression patterns correlated with overall patient survival (2). In the present study, we focus on one of the genes whose high expression correlated with a favorable patient outcome and whose expression pattern helped to define a breast tumor subtype that also expressed the estrogen receptor (ER).<sup>1</sup> This gene encodes a novel member of the *Ras* superfamily of small GTPases that we have designated *REERG*.

The mammalian *Ras* superfamily members serve as molecular switches to regulate a diverse array of cellular functions. These include control of cellular proliferation, differentiation, regulation of the actin cytoskeleton, membrane trafficking, and nuclear transport (3–6). Despite sequence differences between subfamily members, all *Ras*-related GTPases contain five highly conserved domains (G1–G5) and function as guanine nucleotide-dependent molecular switches. For example, the prototypic *Ras* proteins transduce signals for growth and differentiation by alternating between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformational states (7). When in their active GTP-bound state, *Ras*-related proteins interact through their effector domain with a variety of cellular targets to elicit their biological effects.

Recently, several novel *Ras*-related GTPases have been identified that exhibit amino acid sequence features that distinguish them from *Ras* proteins (reviewed in Ref. 8). The *Rheb* GTPases are conserved from human to yeast and are subject to regulated expression in response to a number of stimuli (9, 10). Although the function of *Rheb* in mammalian cells is unclear, the yeast *Rheb* homologue appears to have the ability to regulate cell cycle progression and arginine uptake. The strong amino acid identity of *Rin* and *Rit* proteins with *Ras*, particularly within their effector domains, suggest that *Rit* and *Rin*

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<sup>1</sup> The abbreviations used are: ER, estrogen receptor; HA, hemagglutinin antigen; GTP $\gamma$ S, guanosine 5'-o-(3-thiotriphosphate); Raf, Raf proto-oncogene serine/threonine protein kinase; *Rin*, *Ric* (*Drosophila*)-like, expressed in neurons; GFP, green fluorescent protein.

may share partially overlapping functions with Ras (11–13). Finally, *ARHI* is an imprinted tumor suppressor gene in breast and ovarian carcinomas; expression of *ARHI* in cancer cells inhibited cell growth and was associated with down-regulation of cyclin D1 promoter activity and induction of p21<sup>WAF1/CIP1</sup> (14). Identification of these atypical family members has expanded our understanding of the roles of Ras-superfamily GTP-binding proteins in cell physiology and suggests that they are likely to serve as functionally distinct regulators of as yet to be characterized signaling cascades.

Here we report the identification of a novel member of the Ras superfamily of GTP-binding proteins that was discovered through microarray studies of human breast tumors. Because it is homologous to Ras and regulated in an estrogen-dependent manner, we have termed this gene *RERG* (*ras*-related and *estrogen*-regulated growth inhibitor). *RERG* has biochemical properties characteristic of the Ras superfamily, including an intrinsic ability to bind and hydrolyze GTP; however, overexpression of *RERG* results in a reduced rate of proliferation and a significant inhibition of both anchorage-dependent and anchorage-independent growth.

#### EXPERIMENTAL PROCEDURES

**Microarray Hybridizations and Analysis**—All cDNA microarrays were produced and hybridized as described in Perou *et al.* (1, 15). For the MCF-7 estrogen “add back” experiments, the parental vector-only MCF-7 control cell line (MCF-7-pCDNA3) was grown to 50–70% confluency in RPMI + 10% fetal calf serum + penicillin/streptomycin, at which point the cells were switched to phenol red-free RPMI medium that contained 10% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, UT) plus penicillin/streptomycin for 48 h. Some of these estrogen-deprived cells were then cultured in 10<sup>-8</sup> M  $\beta$ -17-estradiol (Sigma) for 4, 8, and 24 h. For the tamoxifen-treated samples, 70% confluent cultures of MCF-7-pCDNA3 in RPMI + 10% fetal calf serum + penicillin/streptomycin media containing phenol red were treated with either 1 or 6  $\mu$ M tamoxifen (Sigma) for 48 h. mRNA was prepared from each sample using a FastTrack 2.0 mRNA kit (Invitrogen) and compared on 23,000-clone or 44,000-clone cDNA microarrays prepared at Stanford University (1). In the case of the tamoxifen-treated samples, the control untreated cells were labeled with Cy3 and compared with the tamoxifen-treated samples that were labeled with Cy5; for the estrogen add back experiments, the 48-h estrogen-deprived culture was labeled with Cy3 and compared with the estrogen-treated samples (Cy5).

**Cell Culture for Transfections**—NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. MCF-7 human breast carcinoma cells used in *RERG* overexpression studies were maintained in  $\alpha$ -modified Eagle's medium supplemented with 10% fetal calf serum. MCF-7 cells overexpressing wild type *RERG* or vector alone (designated MCF-7-Ha-*RERG* or MCF-7-pCDNA3) were generated by transfecting cells with either pCDNA3-Ha-*RERG* or pCDNA3. Stable MCF-7 transformants were selected for resistance to 0.5 mg/ml G418, and individual clones were isolated using cloning rings. All transfections were performed using LipofectAMINE (Life Technologies, Inc.). Human breast cell lines used in the Northern blot analyses were obtained from ATCC.

**Plasmid Constructions**—The complete *RERG* gene cDNA sequence was determined (GenBank<sup>TM</sup> accession number AF339750). The *RERG* gene was amplified from IMAGE EST clone 28777 using primers that contain *EcoRI*-*NdeI* and *Bam*HI restriction sites and was subcloned into the corresponding sites of the yeast expression vector pWHA, which contains a hemagglutinin (HA) epitope tag, to create pWHA-*RERG*. Next, HA-*RERG* was subcloned into the *EcoRI*-*Bam*HI sites of the human expression vector pCDNA3, resulting in pCDNA3-HA-*RERG*. A full-length *RERG* expression construct for recombinant *RERG* protein production was generated using polymerase chain reaction to introduce a *Bam*HI site immediately upstream of the initiator AUG and an *Xho*I site directly downstream of the 3' stop codon. The polymerase chain reaction product was subcloned into the *Bam*HI and *Xho*I sites of pET-32a (Novagen) to create pET32a-*RERG*. Oligonucleotide site-directed mutagenesis was used to generate the single amino acid substitution mutant RERG<sup>Q64L</sup> (pET32a-*RERG*<sup>Q64L</sup>). For localization studies in live cells, *RERG* in pKH3 was used as a polymerase chain reaction template to generate open reading frame cassettes for subcloning into

pEGFP-C3 (CLONTECH). The cassettes contained 5' *EcoRI* and 3' *Bam*HI restriction enzyme recognition sites and were directionally cloned downstream of and in-frame with the green fluorescent protein (GFP) tag of the vector to express GFP-*RERG* fusion protein.

**RNA Isolation and Northern Blot Analysis**—To analyze *RERG* expression in human breast derived cell lines, RNA was isolated from various cell lines by acid-phenol extraction as described (16). Total RNA (~25  $\mu$ g) was resolved on 1.3% agarose/formaldehyde gel, denatured, and transferred to Hybond-N (Amersham Pharmacia Biotech). [ $\alpha$ -<sup>32</sup>P]dCTP-labeled *RERG* or *GAPDH* cDNA was prepared with the random prime kit (Roche Molecular Biochemicals), purified with Sephadex G50 NICK column (Amersham Pharmacia Biotech), and hybridized to the RNA samples using PerfectHyb (Sigma). A similar process was used to probe a human multiple tissue Northern blot (CLONTECH).

**Recombinant Protein Production and Biochemical Analysis**—The recombinant plasmid pET32a-*RERG* was used for the expression of His<sub>6</sub>-thioredoxin-*RERG* fusion protein as described (17). After purification on nickel nitrilotriacetic acid-Sepharose (Amersham Pharmacia Biotech), *RERG* protein was exchanged into 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM Mg<sup>2+</sup>, 1 mM dithiothreitol, 10% glycerol using a G-25 desalting column (Amersham Pharmacia Biotech) and stored in multiple aliquots at -70 °C. Protein concentrations were determined by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Recombinant *RERG* bound 0.12–0.18 mol of nucleotide/mol as measured by rapid filtration assay (13, 18).

GTP binding to *RERG* was determined by a nitrocellulose filtration assay as described previously (18, 19). *RERG* (1.5  $\mu$ g) or heat-denatured *RERG* (95 °C for 5 min) (1.5  $\mu$ g) was incubated in binding buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 40  $\mu$ g/ml bovine serum albumin, and 2  $\mu$ M GTP $\gamma$ S (0.24  $\mu$ Ci/sample)) containing 1 mM EDTA for 1 min at 22 °C, and the free magnesium concentration was adjusted to 10 mM to initiate nucleotide binding. After incubation at 22 °C for the indicated times, aliquots were withdrawn and filtered immediately through BA85 filters (Schleicher and Schuell), and the amount of bound nucleotide was determined by scintillation counting. Assays to determine the concentration dependence and specificity of binding and monitor guanine nucleotide dissociation were performed as described (13, 18).

GTP hydrolysis was quantitated by thin layer chromatography to resolve GDP and GTP (13, 18). Because the recombinant *RERG* fusion protein was found to contain a contaminating phosphatase activity, unlabeled UTP (2 mM) was added to each reaction as described. GTP hydrolysis assays were performed in buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2  $\mu$ g of *RERG* or *RERG*<sup>Q64L</sup>, 10  $\mu$ M [ $\alpha$ -<sup>32</sup>P]GTP (0.18 Ci/mmol), and either 4 mM UTP or 2 mM UTP and 2 mM GTP. Reactions were incubated at 22 °C for 1 min after which the Mg<sup>2+</sup> concentration was adjusted to 10 mM. The reactions were then incubated at 37 °C for the indicated times, and 1- $\mu$ l aliquots were removed and analyzed as described. The data were quantified using a STORM 850 PhosphorImager (Molecular Dynamics). The percentage of GTP hydrolysis was calculated by dividing the amount of radioactivity in the GDP region by that of the sum of the GTP and GDP regions.

**Western Blot Analysis of Ha-*RERG* and Biochemical Fractionation**—Total cell lysates were prepared by first growing cells to 80% confluence on 100-mm plates. Next, the cells were washed one time with 1 $\times$  phosphate-buffered saline and incubated on ice with 1 ml of radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5) for 30 min with occasional shaking. Cells were then spun for 10 min at 10,000  $\times$  g, and the protein concentration of the lysate was determined by the Bradford assay (Bio-Rad). 40  $\mu$ g of cell lysate was resolved on a 12% SDS-polyacrylamide gel, and Ha-*RERG* expression was analyzed by Western blotting using the anti-HA.11 monoclonal antibody (Berkeley Antibody Co).

Nuclei of MCF-7-pCDNA3 and MCF-7-Ha-*RERG* clones 2B6, 1C2, and 1C4 were isolated from cultures of 80% confluency cells, trypsinized, washed once with phosphate-buffered saline, and incubated in a 10 $\times$  pellet volume of prechilled RSB (10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.4) on ice for 10 min. Cells were then Dounce-homogenized on ice. Next, an aliquot of the cell suspension was examined under a phase contrast microscope for free nuclei. When greater than 90% of the cell suspension was free nuclei, it was centrifuged at 1000  $\times$  g for 3 min at 4 °C. The supernatant was taken as the cytosolic fraction, and the pelleted nuclei were resuspended in 10 volumes of RSB. The nuclei were centrifuged and next resuspended in 10 volumes of RSB. Equal fractions of the nuclear fraction and cytosolic

fraction were loaded onto a 12% SDS-PAGE gel, and Western analysis was performed.

**Localization of GFP-REG in Cultured Cells**—NIH 3T3, MCF-7, or MDA-MB-231 cells were transiently transfected with 1  $\mu$ g of pEGFP or pEGFP-REG using the LipofectAMINE PLUS Reagent (Life Technologies, Inc.) in serum-free medium according to the manufacturer's protocol. Three hours post-transfection, cells were then switched to growth medium, and 20 h post-transfection, cells were viewed in a Zeiss Axiophot fluorescence microscope (63 $\times$  Plan-APOCHROMAT objective) equipped with a cooled charge-coupled device (CCD) camera, and digitized images were captured using MetaMorph<sup>TM</sup> 4.1.4 digital imaging software (Universal Imaging Corp.). GFP expression was detected from 20  $\mu$ g of cell lysate by immunoblotting with anti-GFP monoclonal antibody (CLONTECH) followed by anti-mouse horseradish peroxidase-conjugated antibody and visualized by enhanced chemiluminescence reagent (Pierce).

**Anchorage-dependent and Anchorage-independent Growth Assays**—Growth curves were generated for MCF-7 cells expressing wild type REG protein or vector controls as described (20). In brief, stable transfectants were seeded at  $2 \times 10^4$  cells/well in 6-well plates, then trypsinized and counted in triplicate at the indicated times after seeding. Soft agar growth analyses were performed as described previously (20). Briefly,  $10^4$  MCF-7 cells stably expressing empty vector (control) or REG overexpressing clones 1C4, 1C2, or 2B6 were seeded in duplicate into 0.3% Bacto-agar (Difco) over a 0.6% agar bottom layer, with colonies quantitated after  $\sim$ 20 days.

**Tumorigenicity in Nude Mice**—Tumorigenicity analyses were performed as described previously (20), except that 0.72 mg/60-day-time-release 17- $\beta$ -estradiol pellets (Innovative Research of America) were implanted subcutaneously  $\sim$ 24 h before the injection of cells. MCF-7 cells stably expressing empty vector or REG medium or high expressing clones (1C4 and 2B6, respectively) were injected subcutaneously into 6–8 week athymic nude (BALB/c nu/nu) female mice at  $10^6$  cells/site at two sites per animal (dorsal flanks). Tumor formation was monitored at least twice a week and measured using calipers over the skin of the animal from 9–70 days post-injection.

## RESULTS

**REG Expression in Primary Breast Tumors**—To further investigate the complex variations in gene expression patterns seen within primary human breast tumors, a new analytical method called statistical analysis of microarrays (SAM) (21) was used to analyze microarray data obtained from 78 different human breast tumors (2). This analysis resulted in the identification of 264 genes whose expression correlated with overall patient survival (see Sørbye *et al.* (2), Fig. 7 at genome-www.stanford.edu/breast\_cancer/mopo\_clinical for the complete 264 clone survival associated cluster diagram). Of particular interest were genes in which high expression was associated with favorable patient outcomes and the longest relapse free survival times. A single cluster of genes, which we refer to as the “luminal epithelial/ER+” cluster, contained most of the genes whose high expression was associated with long survival times (2); included within this set of genes was the ER, a known estrogen-regulated gene (LIV-1), and a novel Ras-like GTPase that we call REG for *ras*-related and *estrogen*-regulated growth-inhibitor (Fig. 1A). The mRNA expression pattern of REG across these 78 breast tumors was a statistically significant predictor of patient outcome in a univariate analysis ( $p = 0.0037$ ); however, as would be expected by its expression pattern, it was not an independent predictor of outcome in a multivariate analysis because of its correlation with ER expression ( $p = 0.0001$ ).

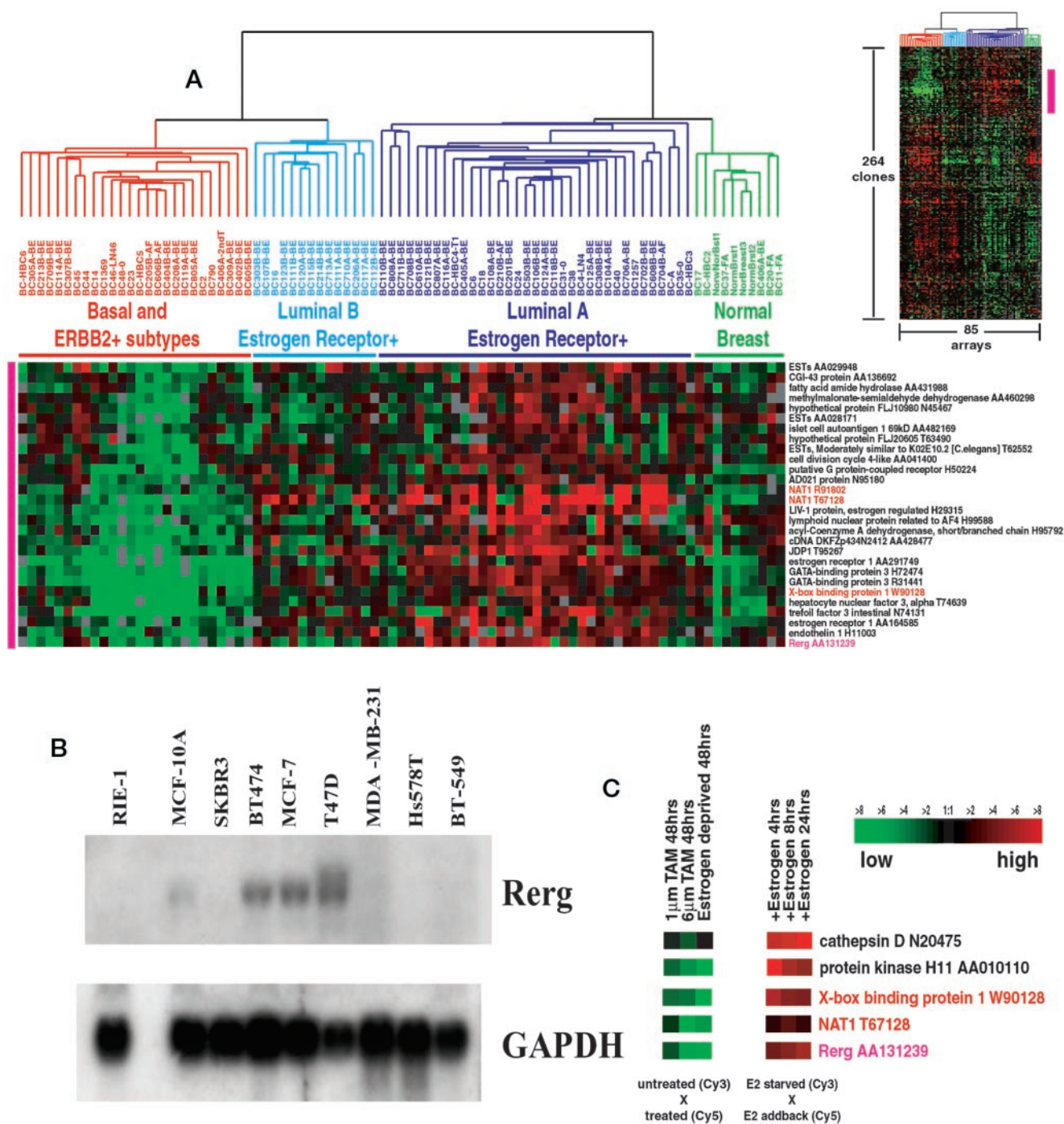
The expression pattern of REG was further investigated in a series of breast-derived cell lines. Northern blot analysis detected REG mRNA in all of the estrogen receptor-positive breast-derived cell lines (MCF-7, MCF-10A, BT474, and T47D) but not in any of the ER-negative cell lines examined (SKBR3, MDA-MB-231, Hs578T, and BT-549 cells) (Fig. 1B).

**Nucleotide Sequence and Expression of the REG Gene**—The REG gene is encoded by an  $\sim$ 2.6-kilobase mRNA that contains a single open reading frame of 600 nucleotides (Gen-

Bank<sup>TM</sup> accession number AF339750). The putative 5' untranslated region includes an in-frame stop codon 87 nucleotides 5' to the putative translation start site, and the sequence surrounding the first ATG fits the consensus sequence for an efficient translation start site (22). The tissue distribution of REG mRNA expression was determined by Northern blot analysis and screening of dbEST; a 2.6-kilobase transcript was detected in all 8 tissues examined (heart, brain, placenta, lung, liver, skin, kidney, and pancreas), with the highest levels of expression in heart, kidney, and pancreas (data not shown). A potentially ubiquitous pattern of expression is supported by Unigene/dbEST expression data (Hs.21594/hypothetical protein MGC15754/accession Number BC007997), which indicates that REG is found in brain, kidney, muscle, prostate, uterus, adrenal gland, aorta, eye, lung, placenta, stomach, and testis. The REG gene is located at 12p12, is contained within two overlapping bacterial artificial chromosome clones (AC007543 and AA022334), and is composed of four exons, the first two of which are separated by a 95-kilobase intron. Analysis of the REG gene genomic sequence identified two potential consensus ER binding sites within the 5' upstream region (data not shown).

**REG Is an Estrogen-responsive Gene**—The location of REG within a gene expression cluster that includes the ER, the high expression of REG in ER-positive breast cell lines, and the presence of two potential ER binding sites within the promoter region suggested that REG might be an estrogen-responsive gene. To evaluate this possibility, we used cDNA microarray analysis and examined REG expression in response to  $\beta$ -estradiol stimulation in the estrogen-responsive MCF-7 cell line (see www4.stanford.edu/MicroArray/SMD for all microarray primary data files). MCF-7 cells grown in estrogen-free media for 2 days were supplemented with  $\beta$ -estradiol ( $1 \times 10^{-8}$  M) for 4, 8, or 24 h before harvesting mRNA and were compared with 48-h estrogen-deprived MCF-7 cultures. As seen in Fig. 1C, REG was rapidly expressed in response to  $\beta$ -estradiol treatment (1.9-fold stimulation after 4 h and 3-fold after 24 h), suggesting that it is a direct target of activated ER. Tamoxifen, an ER antagonist in the breast, acts as an inhibitor of estrogen-induced responses including the regulation of ER-responsive genes. Thus, the effect of tamoxifen (6  $\mu$ M) in the presence of estrogen was examined on global gene expression patterns. As expected for an ER-regulated gene, REG expression was completely repressed by tamoxifen and induced by estrogen although not as dramatically as other known estrogen-regulated genes (see cathepsin D and protein kinase H11 for examples of how known ER-responsive genes behaved in these experiments) (Fig. 1C).

**REG Is a GTP-binding Protein with Intrinsic GTPase Activity**—The predicted amino acid sequence of the REG protein is encoded by an open reading frame of 600 base pairs that codes for a 199-amino acid protein that is 50 and 47% identical to the *Dictyostelium* Ras-D and Ras-S proteins respectively, and 44 to 45% identical to human Rit, TC21/R-Ras-2, M-Ras/R-Ras-3, Rin, and Rap1b (Fig. 2). Although REG shares significant sequence identity and organization with Ras, it also contains a number of unique features. First, REG has an alanine at the position corresponding to the glycine at position 12 of Ki-Ras. Substitution of this residue with alanine results in the constitutive activation of Ras (23). Another unique feature of REG is that it lacks any known COOH-terminal prenylation motif, indicating that it is not subject to post-translational prenylation (24, 25). Prenylation facilitates the association of Ras with the plasma membrane, and nonprenylated mutants of Ras are rendered completely inactive. Instead, stretches of basic residues are found in the REG COOH-terminal region. These unique



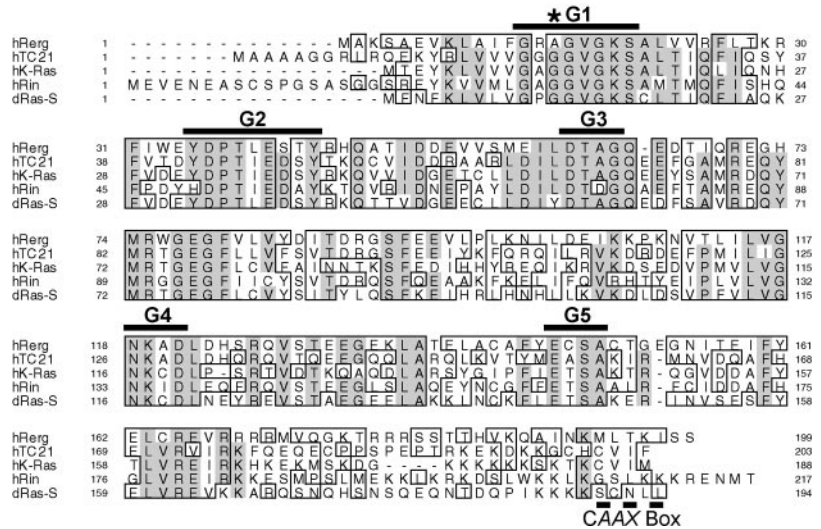
**FIG. 1. mRNA expression of RERG across 78 breast tumors, 3 fibroadenomas, and 4 normal breast samples.** *A*, the RERG gene (identified in pink) expression pattern is part of the luminal epithelial/ER-positive subset of genes as defined in Sørlie *et al.* (2). The luminal epithelial/ER-positive portion of a hierarchical-cluster diagram is shown and was taken from the larger cluster diagram formed when using the 264 cDNA clones that statistically correlated with overall patient survival (see Fig. 7 at [genome-www.stanford.edu/breast\\_cancer/mopo\\_clinical](http://genome-www.stanford.edu/breast_cancer/mopo_clinical) for the complete SAM 264 cluster diagram). The GenBank<sup>TM</sup> accession numbers next to each gene name represents the accession number for the actual cDNA clones that were spotted on the microarrays. *B*, Northern blot analysis of endogenous RERG expression in human breast-derived cell lines and rat intestinal epithelial cells (RIE-1). *C*, summary of estrogen modulation microarray experiments. Untreated MCF-7 cells grown in phenol red-containing media (Cy3) were compared with either 48-h estrogen-deprived cells (Cy5) or 48-h tamoxifen (TAM)-treated cells (Cy5) (*left panel of C*). 48-h estrogen-deprived cells (Cy3) were compared with cells that were first deprived of estrogen for 48 h and then stimulated with estrogen for 4, 8, or 24 h (Cy5) (*right panel of C*). Selected gene expression results are shown, with the behavior of two known estrogen-regulated genes (cathepsin D and protein kinase H11 (36, 37)) displayed along with RERG and two other genes (X-box-binding protein 1 and NAT1) that were also contained within the luminal epithelial/ER-positive expression cluster. The color scale, which represents the fold change observed, for *A* (relative to the median expression) and *C* is present at the lower right. All microarray data can be obtained at [genome-www4.stanford.edu/MicroArray/SMD](http://genome-www4.stanford.edu/MicroArray/SMD).

COOH-terminal features of RERG are similar to those of Rit and Rin (11–13).

The GTP binding activity of recombinant RERG was assayed using [<sup>35</sup>S]GTP $\gamma$ S, and bound GTP was separated by rapid filtration. As seen in Fig. 3A, native recombinant RERG but not

heat-inactivated RERG binds GTP $\gamma$ S rapidly in a Mg<sup>2+</sup>-dependent manner upon incubation under standard nucleotide exchange conditions. As observed with other GTPases, the association of guanine nucleotides with RERG was greatly affected by the concentration of magnesium ions. Replacement of

**FIG. 2. Comparison of the amino acid sequences of the predicted human proteins RERG, TC21, Ki-Ras, Rin, and Dictyostelium Ras-S.** The alignment was performed with the CLUSTAL W1.6 program (38). *Hyphens* represent gaps introduced for optimal alignment. *Numbers* are residue numbers. Amino acid residues that are identical in at least four of the five proteins in the alignment are placed in *shaded boxes*, and amino acid similarity is indicated by *boxes*. Consensus sequences for GTP binding regions (G1-G5) (*heavy line*) and the position of conserved CAAX motif (*dashed line*) are labeled. The position of alanine 15 in RERG is indicated with an *asterisk*. *h-*, human; *d*, *Dictyostelium*.



MgCl<sub>2</sub> in the reaction with EDTA completely abolished GTPγS binding (data not shown). The binding of both GDP and GTPγS was time- and concentration-dependent (Fig. 3B). The ability of various nucleotides to compete for [<sup>35</sup>S]GTPγS binding to RERG was also examined and showed that RERG is a specific guanine nucleotide-binding protein, since an excess (20-fold) of unlabeled GTPγS, but not CTP, UTP, or ATP, could compete for [<sup>35</sup>S]GTPγS binding (Fig. 3C). These GTP binding properties are shared with those of Ras and all other Ras superfamily GTPases.

The RERG dissociation curves follow a single exponential pattern, as would be expected for a single class of nucleotide binding site (Fig. 3D). The half-life for [<sup>3</sup>H]GDP release from RERG was extremely slow and, therefore, difficult to assess accurately in this study (half-life > 60 min). Surprisingly, RERG released GTPγS quite rapidly, with a half-life of ~7 min. This rapid rate of release is in contrast to the slower rate of release seen for the majority of Ras-related proteins.

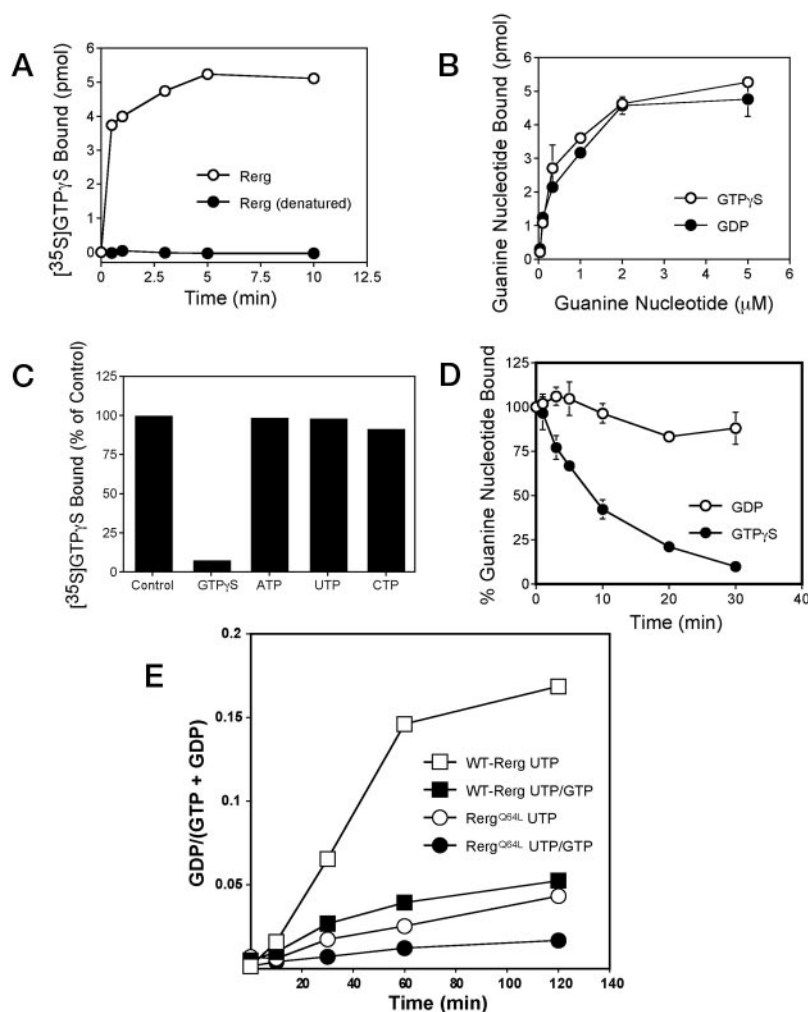
To determine whether RERG was able to hydrolyze GTP to GDP, recombinant RERG was incubated with [α-<sup>32</sup>P]GTP, and the products were analyzed by thin-layer chromatography. As seen in Fig. 3E, recombinant RERG was capable of hydrolyzing [α-<sup>32</sup>P]GTP in the presence of an excess of unlabeled UTP. When UTP was replaced with GTP, we did not observe the production of GDP, suggesting that the hydrolysis represents RERG-mediated GTPase activity. Further proof that the GTPase activity is intrinsic to the RERG protein comes from analysis of the RERG mutant, RERG<sup>Q64L</sup>. The analogous mutation in Ras (Q61L) results in a protein with decreased GTPase activity, and this mutation is found in constitutively activated and transforming forms of Ras (26). The initial characterization of RERG<sup>Q64L</sup> found it to have guanine nucleotide binding properties that were similar to the wild type protein; however, the GTPase activity of this mutant was greatly reduced (Fig. 3E).

**Subcellular Localization of RERG**—To determine the subcellular localization of RERG, we transiently expressed a GFP-tagged RERG fusion protein in the MCF-7 and MDA-MB-231 human breast tumor-derived cell lines and in NIH 3T3 mouse fibroblasts. As shown in Fig. 4A, transient transfection with the wild type GFP-RERG vector led to the production of an intact GFP-RERG fusion protein, as determined by anti-GFP Western blotting. Epifluorescence microscopy of live cells transiently expressing GFP-RERG revealed a uniform and diffuse distribution throughout the cell cytoplasm, including some signal in the nucleus (Fig. 4B). A similar expression pattern and distribution was detected in MCF-7 cells stably expressing

HA-epitope tagged RERG and in a series of additional cell lines transiently expressing GFP-RERG (data not shown). No cells were observed that exhibited a plasma membrane localization of RERG, which is consistent with the lack of a COOH-terminal prenylation signal sequence. This distribution is similar to that seen in cells expressing GFP alone (Fig. 4B). The distribution of HA-tagged RERG was further examined using biochemical fractionation and Western blot analysis and showed that HA-RERG was present in the cytosolic fraction of MCF-7 cells that were stably expressing HA-RERG fusion proteins (Fig. 4C).

**REG Is a Growth Inhibitor in MCF-7 Breast Carcinoma Cells**—The core effector domain of Ras (residues 32–40) is critical for Ras interactions with Raf and other effector proteins (4). RERG shares strong, but incomplete, identity with Ras in this region, suggesting the possibility that RERG may interact with effectors of Ras (Fig. 2). To address this possibility, we characterized the potential interaction between RERG and a panel of known Ras effectors. No interactions between RERG and Raf, Ral guanine nucleotide dissociation stimulator (Ral-GDS), phosphoinositide 3-kinase, Rin1, or Rin2 were detected using a yeast two-hybrid assay (data not shown). Consistent with the lack of interaction of RERG with Ras effectors, RERG expression did not result in the appearance of foci of transformed cells when tested in a NIH 3T3 focus-formation assay (data not shown). Furthermore, unlike what was seen for other Ras-related proteins that lack COOH-terminal CAAX motifs (27), no cooperation with activated Raf to cause focus-formation was seen. Finally, we did not observe any significant inhibitory effects of wild type or mutant RERG expression on the transformation of NIH 3T3 cells by H-Ras<sup>61L</sup> (data not shown).

When we closely examined the expression of *REG* across the breast tumors presented in the study by Sørlie *et al.* (2) and compared this expression pattern with the expression of the “proliferation” cluster set of genes (1, 15, 28, 29), we observed that the tumors that most highly expressed *REG* tended to be the least proliferative and the ones with low to no *REG* expression tended to be the most proliferative (see genome-www.stanford.edu/breast\_cancer/mopo\_clinical). The lack of *REG* expression within many breast carcinomas that are highly proliferative suggests that RERG may function as an inhibitor of growth. To address this possibility, we generated MCF-7 cell lines stably overexpressing wild type RERG and evaluated the consequences on growth *in vitro* and *in vivo*. We chose MCF-7 cells for these analyses because they were positive for *REG* mRNA expression and are an ER-positive cell line. Clonal populations of MCF-7 cells stably transfected with an expression vector encoding a HA epitope-tagged version of

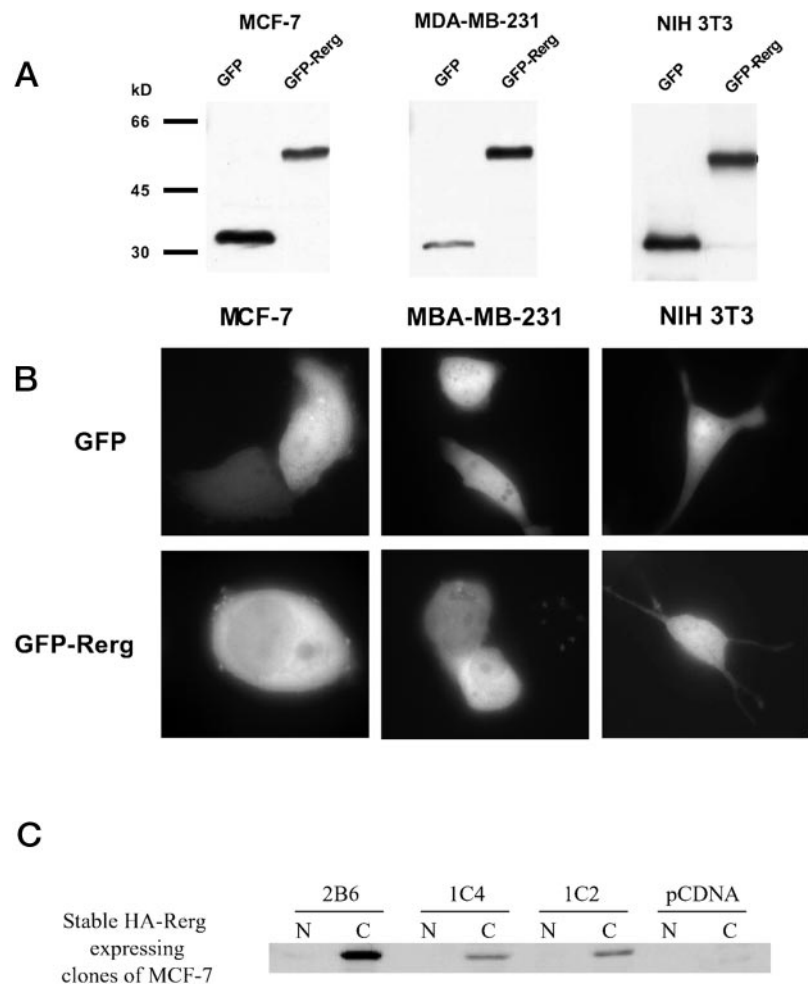


**FIG. 3. Biochemical properties of RERG.** *A*, the time dependence of guanine nucleotide binding to RERG was determined by incubating 1.5  $\mu\text{g}$  of RERG (open circle) or heat-denatured RERG (closed circle) with 2  $\mu\text{M}$  [ $^{35}\text{S}$ ]GTP $\gamma$ S and 1 mM EDTA for 1 min. The free  $\text{Mg}^{2+}$  was then adjusted to 10 mM to initiate GTP $\gamma$ S binding. At the indicated times, the amount of [ $^{35}\text{S}$ ]GTP $\gamma$ S bound was determined by rapid filtration and scintillation counting. *B*, the concentration dependence of guanine nucleotide binding to RERG was determined by incubating EDTA-treated RERG (1.5  $\mu\text{g}$ ) with the indicated concentration of either [ $^3\text{H}$ ]GDP (closed circle) or [ $^{35}\text{S}$ ]GTP $\gamma$ S (open circle). Free  $\text{Mg}^{2+}$  was adjusted to 10 mM, and radiolabeled nucleotide binding was determined by rapid filtration. *C*, the specificity of RERG guanine nucleotide binding was determined by incubating RERG (1.5  $\mu\text{g}$ ) with 2  $\mu\text{M}$  [ $^{35}\text{S}$ ]GTP $\gamma$ S (Control) or 2  $\mu\text{M}$  [ $^{35}\text{S}$ ]GTP $\gamma$ S and 40  $\mu\text{M}$  indicated non-radiolabeled ribonucleotide with 1 mM EDTA for 1 min. Free  $\text{Mg}^{2+}$  was then adjusted to 10 mM, and the bound [ $^{35}\text{S}$ ]GTP $\gamma$ S at 10 min was determined by rapid filtration. The amount of radionucleotide bound in the absence of non-radiolabeled ribonucleotide was set as 100%. *D*, the guanine nucleotide dissociation rates from RERG were determined by incubating RERG (1.5  $\mu\text{g}$ ) with either 2  $\mu\text{M}$  [ $^3\text{H}$ ]GDP (open circle) or [ $^{35}\text{S}$ ]GTP $\gamma$ S (closed circle) and 1 mM EDTA for 1 min. The free  $\text{Mg}^{2+}$  was then adjusted to 10 mM to initiate binding. To initiate dissociation, a 100-fold molar excess of unlabeled GDP or GTP $\gamma$ S was added, and the exchange of radiolabeled nucleotide was measured as described under "Experimental Procedures." The amount bound at 0 min was set to 100%. *E*, the ability of RERG and RERG<sup>Q64L</sup> to hydrolyze GTP was determined as described under "Experimental Procedures." RERG (squares) and RERG<sup>Q64L</sup> (circles) (2  $\mu\text{g}$ ) were incubated with [ $\alpha$ - $^{32}\text{P}$ ]GTP (10  $\mu\text{M}$ ; 2 Ci/mmol) and 1 mM EDTA in the presence of either 4 mM UTP (open symbols) or 2 mM UTP and 2 mM GTP (closed symbols) for 1 min. The free  $\text{Mg}^{2+}$  was then adjusted to 10 mM to initiate binding, and 1- $\mu\text{l}$  aliquots were removed and analyzed by thin layer chromatography. All values are the average of duplicate points and are representative of experiments repeated at least three times. WT, wild type.

wild type RERG were generated; empty pCDNA3 vector-transfected cells were also isolated as controls for these analyses. Western blot studies with anti-HA antibodies verified expression of HA-RERG in three clonal RERG-overexpressing isolates, with the highest expression seen in 2B6, then 1C4, and lowest expression in 1C2 (Fig. 5A).

We first assessed the consequences of constitutively high RERG expression on the rate of proliferation when the cultures were maintained on plastic. All three clonal cell lines expressing HA-RERG showed reduced rates of cell proliferation when compared with the vector-transfected control cell line (Fig. 5B). A similar growth inhibition was observed with MCF-7 cells when RERG expression was transient-induced under the control of an inducible promoter (data not shown). Second, we determined if RERG overexpression affected an-

chorage-independent growth potential of MCF-7 cells. We determined that MCF-7 cells expressing HA-RERG showed greatly reduced colony-forming efficiency (50–80% reduction) in soft agar when compared with the control vector only MCF-7 cells (Fig. 5C). Finally, we determined if the *in vivo* tumorigenic potential of MCF-7 cells was influenced by RERG overexpression. For these analyses, we inoculated the cell lines subcutaneously into nude mice and implanted estrogen pellets for timed release of estrogen. Tumors grew at each site injected with empty vector control MCF-7 cells with a latency of  $\sim$ 52 days (Table I). Tumors were also observed in mice injected with low HA-RERG-expressing 1C4 cells with a similar latency. In contrast, mice injected with the high HA-RERG-expressing 2B6 clone remained tumor-free for an additional month (Table I).



**FIG. 4. Subcellular distribution of RERG in living cells.** *A*, Western blot analysis of MCF-7, MDA-MB-231, and NIH 3T3 cells using an anti-GFP monoclonal antibody (CLONTECH) shows that GFP-RERG is expressed at the predicted molecular weight for the fusion protein. *B*, RERG is expressed in the cytoplasm and nucleus of MCF-7, MDA-MB-231, and NIH 3T3 cells when ectopically expressed. The cell lines were transiently transfected with DNA constructs encoding unfused GFP (*top panels*) or GFP-RERG (*bottom panels*), and the live cells were examined by epifluorescence microscopy as described under "Experimental Procedures." *C*, biochemical fractionation of MCF-7 cell clones that stably express HA-RERG into nuclear (*N*) and cytoplasmic (*C*) fractions shows that HA-RERG is predominantly located in the cytoplasmic fraction.

**DISCUSSION**

We employed microarray analyses to identify genes whose expression correlated with a favorable prognosis for breast cancer patients. One gene identified represents a novel *ras*-related gene that we have designated *RERG* (*ras*-related and *estrogen*-regulated growth inhibitor). We determined that *RERG* expression was decreased or lost in a significant percentage of primary human breast tumors that showed a poor clinical prognosis and was regulated by estrogen stimulation *in vitro*. Like Ras, RERG protein can bind and hydrolyze GTP, indicating that RERG also functions as a GTPase. Unlike Ras, RERG lacks any known COOH-terminal prenylation signal sequence and exhibited a cytosolic rather than plasma membrane subcellular location. RERG did not interact with effectors of Ras, indicating that RERG function is likely distinct from that of Ras. Finally, in contrast to the growth-promoting activity associated with Ras and other Ras-related proteins, RERG overexpression inhibited the growth of breast tumor cells *in vitro* and *in vivo*. When taken together, these observations support an important role for the loss of RERG function in the development of breast cancer.

We have identified a novel estrogen-regulated member of the Ras superfamily of small GTP-binding proteins. Analysis of the *RERG* cDNA sequence indicates that it encodes a protein of 199 amino acids with a deduced molecular mass of 22,607 Da. The regions with highest homology between RERG and other Ras family members corresponds to the five regions of Ras involved in GTP binding (G1-G5) (Fig. 2) (30). The core consensus sequence of the G1 region, GX<sub>4</sub>GK(S/T) (Ras residues 10–17) is present in RERG; however, RERG contains an alanine at the

position corresponding to glycine 12 of Ras and the majority of Ras superfamily proteins. Interestingly, *ARHI/NOEY2* also harbors a naturally occurring "activating" substitution at this position. A G12A substitution in Ras causes an impairment of intrinsic GTPase activity and results in a constitutively activated and transforming Ras protein (23). We found, however, that RERG possesses an intrinsic GTPase activity that was impaired when a mutation analogous to the Ras<sup>Q61L</sup> mutation, which abolishes the intrinsic and GTPase-activating protein-stimulated GTPase activity of Ras and other Ras family GTPases, was introduced into RERG. Thus, despite this structural difference from Ras, it is possible that RERG is not constitutively active and GTP-bound. It will be important to determine whether there are specific guanine nucleotide exchange factors and/or GTPase-activating protein that regulate RERG function and if RERG-GTP levels are regulated by extracellular stimulus-mediated signaling pathways.

Although RERG shares strong sequence identity with the core effector domain of Ras, we determined that RERG did not bind to Raf and other effectors of Ras. This observation together with the lack of transforming activity in NIH 3T3 cells argues that RERG function is mediated by interactions with downstream targets distinct from those of Ras. Although many Ras superfamily GTPases have been described as positive regulators of cell growth and differentiation (8), others including Rap1 and ARH1 have been implicated as negative regulators of Ras-mediated pathways (14, 31). Our studies suggest that RERG functions as a negative growth regulator without directly affecting Ras signaling. Defining RERG-mediated signaling cascades will require further investigation.

**FIG. 5. Inhibition of anchorage-dependent and anchorage-independent growth of MCF-7 cells by the expression of RERG.** *A*, cytosolic extracts (40  $\mu$ g of protein) from MCF-7 cells stably expressing empty vector or Ha-RERG overexpressing clones 1C2, 1C4, or 2B6 were subjected to Western blot analysis using an anti-HA monoclonal antibody. *B*, growth curves for MCF-7 cells stably expressing empty vector or Ha-RERG clones were generated as described under "Experimental Procedures." Cells were counted from triplicate wells every day for 7 days. Data shown are representative of three independent experiments. *C*, MCF-7 clones stably expressing empty vector or Ha-RERG isolates 1C4, 1C2, or 2B6 were seeded in duplicate into 0.3% soft agar over a 0.6% agar bottom layer and maintained at 37 °C. Colonies were quantitated after ~20 days. Data shown are expressed as a percent of colonies formed by control vector-transfected cells and are an average of three independent experiments.

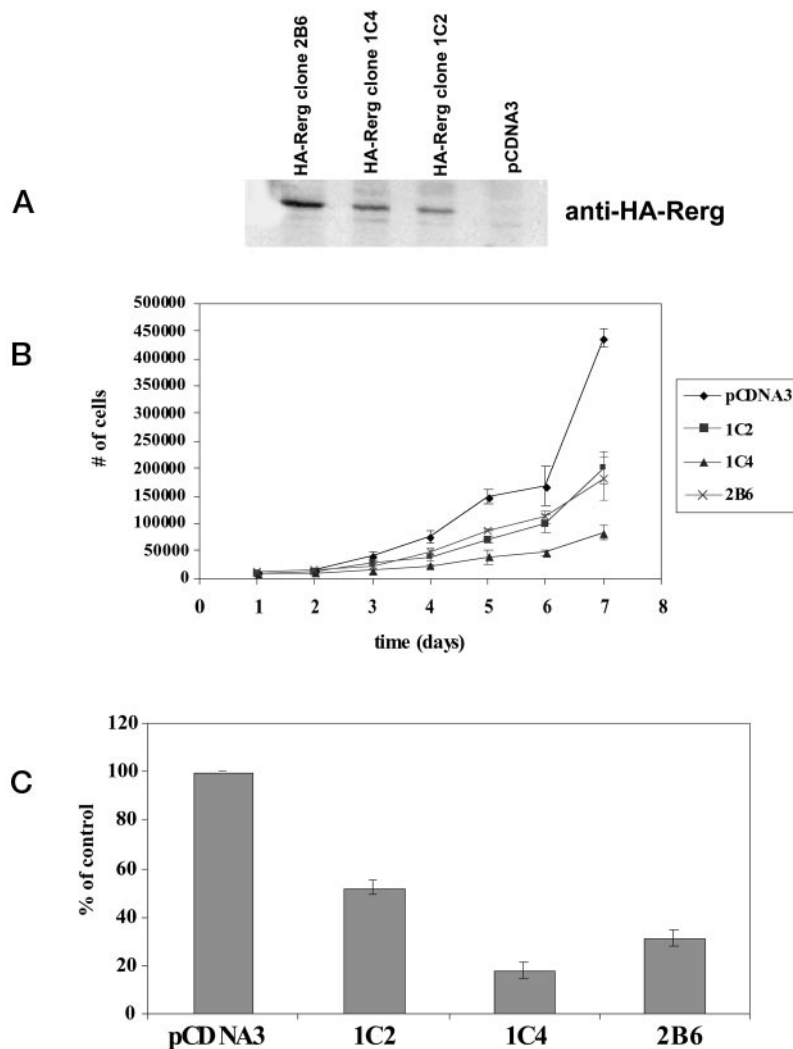


TABLE I

*Suppression of MCF-7 cell tumor formation in nude mice by Rerg expression*

6–8 week old athymic (nude) mice implanted with estrogen pellets were injected with MCF-7 cells stably expressing empty vector or Rerg-overexpressing clones 1C4 or 2B6. Tumor formation was monitored approximately twice a week from 9–70 days post-injection.

Day post-injection	Vector	Rerg 1C4	Rerg 2B6
9	0/4	0/4	0/4
17	0/4	0/4	0/4
22	0/4	0/4	0/4
31	0/4	0/3	0/4
37	0/4	0/3	0/4
43	0/4	1/3	0/4
47	0/4	1/3	0/4
52	2/4	1/3	0/4
57	2/4	2/3	0/4
65	2/4	2/3	0/4
70	2/4	2/3	0/4

Most Ras-related proteins are membrane-associated and specific membrane localization is both essential and central to their biological activity (25, 32). Membrane binding generally requires the post-translational addition of a COOH-terminal isoprenyl group by a mechanism that involves the prenyltransferase recognition of conserved cysteine motifs (prenylation) as well as other CAAX-signaled posttranslational modifications (24). One of the distinguishing structural features of RERG is a unique COOH-terminal sequence that contains stretches of basic residues but lacks a consensus motif for lipid modification

(Fig. 2). Indeed, epifluorescence microscopy revealed that RERG is predominately localized in the cytoplasm when expressed in a variety of cell types including breast derived cell lines (Fig. 4). In this regard, RERG is similar to the recently described Rem/Rad/Gem/Kir proteins, which lack a COOH-terminal CAAX motif and appear to be localized to the cytosol (18, 33). Additional studies including the localization of endogenous RERG protein and studies to identify RERG-interacting proteins, will be necessary before the significance of this unique cellular distribution can be fully understood.

An important finding of this study is that the expression of RERG in MCF-7 breast carcinoma cells can be modulated by estrogen and anti-estrogen treatment (Fig. 1C). Estradiol stimulation markedly and rapidly increased the expression of RERG in a time-dependent manner, whereas tamoxifen or estrogen deprivation potently repressed RERG expression to no expression. Because two consensus estrogen response elements were found within the promoter region of the RERG gene, we believe RERG to be an estrogen-regulated gene. Consistent with this hypothesis is our finding that RERG expression demonstrated a high degree of correlation with ER in both breast tissue-derived cell lines and primary tumors (Fig. 1, A and B). The expression of the estrogen receptor has important implications for the biology of breast carcinomas. Patients with tumors that express ER have a longer disease-free interval and overall survival than those patients with tumors that lack ER expression (34, 35). The marked physiological and phenotypic differences between ER-positive and ER-negative breast tu-



mors have been hypothesized to be due to differences in gene expression between these two tumor types. Therefore, there is a great need to identify estrogen-responsive genes within primary breast tumors and define their role in tumorigenesis. These studies indicate that REG may be of biological and/or clinical importance in breast cancer and suggest an important role for REG in the physiological response of breast epithelial cells to estrogen.

The role of REG in breast tumor growth was evaluated in several experimental models. The overexpression of REG in MCF-7 cells markedly inhibited their anchorage-dependent growth rates under standard tissue culture conditions (Fig. 5B). More importantly, elevated REG expression significantly reduced colony formation in soft agar (Fig. 5C) and inhibited tumor formation in nude mice (Table I). Taken together, these results suggest that loss of REG expression may facilitate transformation of breast luminal cells. It should be noted that the phenotypes caused by overexpression of REG in MCF-7 cells are entirely consistent with its expression pattern seen across the set of 78 breast tumors presented in Fig. 1; the group of tumors that most highly expressed the luminal epithelial/ER-positive set of genes (that contains REG and termed "luminal subtype A"), were on average some of the least proliferative tumors (1, 2). These studies suggest that REG may function as a negative growth regulator in breast epithelial cells. Whether this inhibition depends on the cellular context in which REG is expressed and the identification of the signal transduction cascades that are impacted remains to be determined.

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