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Identification of a Novel Human Rho Protein with Unusual Properties: GTPase Deficiency and In Vivo Farnesylation

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We have identified a human Rho family protein, RhoE, which has unusual structural and biochemical properties that suggest a novel mechanism of regulation. Within a region that is highly conserved among small GTPases, RhoE contains amino acid differences specifically at three positions that confer oncogenicity to Ras (12, 59, and 61). As predicted by these substitutions, which impair GTP hydrolysis in Ras, RhoE binds GTP but lacks intrinsic GTPase activity and is resistant to Rho-specific GTPase-activating proteins. Replacing all three positions in RhoE with conventional amino acids completely restores GTPase activity. In vivo, RhoE is found exclusively in the GTP-bound form, suggesting that unlike previously characterized small GTPases, RhoE may be normally maintained in an activated state. Thus, amino acid changes in Ras that are selected during tumorigenesis have evolved naturally in this Rho protein and have similar consequences for catalytic function. All previously described Rho family proteins are modified by geranylgeranylation, a lipid attachment required for proper membrane localization. In contrast, the carboxy-terminal sequence of RhoE predicts that, like Ras proteins, RhoE is normally farnesylated. Indeed, we have found that RhoE is farnesylated in vivo and that this modification is required for association with the plasma membrane and with an unidentified cellular structure that may play a role in adhesion. Thus, two unusual structural features of this novel Rho protein suggest a striking evolutionary divergence from the Rho family of GTPases.

Members of the Ras superfamily of regulatory proteins, which includes the Ras, Rho, Rab, and Arf subfamilies, share two important biochemical features that are essential for their normal functions. First, these proteins are able to bind to and hydrolyze GTP, thereby providing their switching capability (6, 7). Second, these proteins undergo a characteristic posttranslational modification that results in the attachment of a lipid group (isoprenoid) to the carboxyl terminus which is required for association with cellular membranes (16). Both of these properties are determined by primary sequence motifs that have been highly conserved evolutionarily and whose importance has been confirmed through mutational analysis (7, 16). For Ras proteins, the most extensively studied of the GTPases thus far, the identification in tumors of activating mutations that impair GTPase activity revealed a few highly conserved amino acids that are critical for normal hydrolytic function (5, 33, 46). Conservation of these amino acids is not limited to the Ras family proteins but extends to the large variety of guanine nucleotide-binding proteins (G proteins) with diverse biological functions. In fact, activating mutations similar to those seen in Ras have been detected in the Gα subunits of heterotrimeric G proteins in several human tumors (28, 29). Such observations together with structural data derived from crystallographic analyses of several GTPases (23, 37) have led to the identification of a highly conserved catalytic site that is responsible for GTP binding and hydrolysis. Moreover, primary structure comparisons of the dozens of GTPases across the evolutionary spectrum have defined a conserved structure within the predicted catalytic site that is determined by several nearly invariant amino acids (7). These include positions analogous to Ras amino acids 12, 59, and 61, each of which contributes to the structure of a functional catalytic site in Ras and is required for normal GTP hydrolysis, as revealed by mutational analysis. For the Rho proteins, a growing family of Ras-related GTPases that regulate the actin cytoskeleton, mutational analysis has revealed a similar critical role for these highly conserved amino acids in normal GTPase activity, again confirming the importance of structural conservation for the GTP hydrolysis mechanism (15, 44, 50–52).

In addition to a conserved structure for GTP hydrolysis, all members of the Ras and Rho families contain a so-called CAAX motif at their carboxyl termini (C, cysteine; A, aliphatic amino acid; X, any amino acid). The CAAX motif is a peptide signal for three types of posttranslational modification, namely, prenylation, proteolysis, and methylation (16). For both Ras and Rho proteins, the presence of this CAAX motif is crucial for normal plasma membrane localization and biological activity (16).

CAAX-containing proteins are either farnesylated or geranylgeranylated, depending on the identity of the carboxy-terminal amino acid (X). Early studies pointed to some simple rules for CAAX prenylation specificity (34, 39). Proteins will be geranylgeranylated if X is leucine or phenylalanine, while a farnesyl group will be added if X is methionine, serine, alanine, cysteine, or glutamine. The importance of the last amino acid in directing prenylation specificity is supported by the observation that changing this position in Ras from nonleucine residues to leucine residues results in geranylgeranylation instead of farnesylation (3, 43). Unlike the Ras proteins, which contain a CAAX motif that specifies farnesylation, the Rho proteins identified thus far, including RhoA, RhoB, RhoC, Rac1, Rac2, and CDC42, all end with leucine or phenylalanine and are thus geranylgeranylated (1, 25, 54). Notably, RhoB (carboxy-termi-

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nal leucine) is found in both farnesylated and geranylgeranylated forms, although it has recently been demonstrated that both of these modifications of RhoB can be produced by the geranylgeranyltransferase enzyme (3), consistent with the notion that the carboxy-terminal amino acid of the GTPase determines specificity for the prenylase.

In this report, we describe the identification of a novel Rho family protein, RhoE, which, despite strong similarity to other small GTPases, exhibits specific structural differences in the highly conserved domains that determine both GTPase activity and prenylation. RhoE has an unusual structure within the nucleotide-binding site and is consequently unable to hydrolyze GTP. Unlike previously characterized GTP-binding proteins, which oscillate between inactive GDP-bound and active GTP-bound forms (7), this Rho protein appears to be normally maintained in a GTP-bound state, suggesting that it may not be regulated by a conventional cycling mechanism. In addition, the carboxy-terminal sequence of RhoE predicts that, unlike other Rho proteins, RhoE undergoes the farnesylation characteristic of Ras proteins.

MATERIALS AND METHODS

Interaction trap screening and cDNA cloning. To conduct a yeast interaction trap screen with p190, the complete rat p190 coding sequence (45) was fused in frame to the LexA DNA-binding domain in a yeast expression vector (17) and used as bait to screen a HeLa cell cDNA expression library. Positive clones were identified by expression of interaction-dependent reporter genes and retested with a variety of unrelated baits to confirm the specificity of the interaction. Of 7×10^7 clones screened, 30 positive clones which specifically interacted with p190 and not with other unrelated baits that were tested were identified. Of these, the Rho-like clone was isolated once. Following subcloning and sequencing of the positive library clone insert, the purified insert was ^{32}P labeled by random priming and used as a hybridization probe to screen a cDNA library under standard high-stringency screening conditions. A total of 5×10^5 cDNA clones from a human fetal brain library (Stratagene) were screened, and 4 clones that remained positive upon multiple rescreening were isolated, subcloned into pBluescript (Stratagene), and sequenced by the dideoxy method, using Sequenase (U.S. Biochemical Corp.). The sequence was found to correspond exactly to that of the original HeLa cell clone.

Northern (RNA) blot analysis. A Northern blot containing, per lane, 2 μg of poly(A)$^+$-selected RNA from each of several human tissues was obtained from Clontech. High-stringency hybridization was performed under standard conditions, using a ^{32}P-labeled RhoE cDNA clone.

Nucleotide binding assays. RhoA and RhoE coding sequences were fused in frame to glutathione *S*-transferase (GST) in pGEX vectors (Amrad) by PCR, expressed in bacteria, and purified with glutathione-agarose. Protein expression was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining. To load proteins with radiolabeled GTP, each protein (5 to 20 μg) was incubated with [α-^{32}P]- or [γ-^{32}P]GTP (10 μCi; 6,000 Ci/mmol; DuPont NEN) in 50 mM Tris-HCl (pH 7.5)–50 mM NaCl–5 mM EDTA–0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–0.1 mM dithiothreitol (DTT)–10 μM ATP (100 μl) at 37°C for 10 min. The rate of nucleotide dissociation from preloaded proteins was determined by filter binding to BA85 nitrocellulose (0.45-μm pore size) following incubation of 2 μg of protein in a buffer (50 μl) containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM DTT, 1 mM GTP, and either 5 mM EDTA (low Mg^{2+}) or 5 mM $MgCl_2$ (high Mg^{2+}) at 37°C for the indicated times. Filters were washed twice with 4 ml of 50 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$ each time.

GTPase and GAP assays. GTP hydrolysis of γ-^{32}P-loaded proteins was performed in a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 10 μM GTP, and 1 mM DTT (50 μl); 200 ng to 1 μg of labeled protein was typically used per assay. To measure GTPase activity by thin-layer chromatography (TLC), fusion proteins bound to glutathione-agarose beads were loaded with [α-^{32}P]GTP as described above. After incubation at 37°C, the beads were washed three times in wash buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT, and 1 mg of bovine serum albumin (BSA) per ml. Hydrolysis was carried out at 37°C for the indicated times and stopped by the addition of ice-cold wash buffer followed by repeated washing in the same buffer. Bound nucleotide was eluted by incubation of the beads in 1% SDS–20 mM EDTA at 65°C for 5 min. Eluted samples were spotted on polyethyleneimine-cellulose plates (EM Sciences), and the released nucleotides were resolved by TLC in 0.75 M KH_2PO_4 (pH 3.4) and visualized by autoradiography. GTPase-activating protein (GAP) assays were performed with either purified, baculovirus-produced p190 (44) or a purified GST-CDC42 GAP fusion protein (40 nM) in a filter binding assay as previously described (44).

Phosphate labeling and immunoprecipitation. PCR cloning was used to insert the RhoA and RhoE coding sequences into a modified Rc/CMV vector that places an influenza virus hemagglutinin (HA) epitope at the amino terminus. Expression of the fusion proteins was confirmed by transfection of COS cells followed by Western blotting (immunoblotting) with the anti-HA monoclonal antibody 12CA5. COS transfections were performed with DEAE-dextran (10 μg of DNA per 10-cm-diameter plate), and cells were subjected to a 45-s 10% dimethyl sulfoxide shock 4 h after transfection. Cells were labeled with $^{32}P_i$ (0.5 mCi/ml) 24 h posttransfection by incubation in low-phosphate Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum that had been dialyzed against 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–0.9% NaCl as previously described (14). The cells were lysed and clarified, and precleared lysates were incubated with monoclonal antibody 12CA5 and protein A-Sepharose beads as previously described (14) except that 100 μM ATP was added to the lysis buffer to reduce nonspecific binding of nucleotide. Following extensive washing of the antibody-antigen complexes, associated nucleotide was eluted by incubation in 20 mM HEPES (pH 7.0)–100 mM NaCl–10 mM EDTA–2 mM GTP–2 mM GDP at 37°C for 60 min, resolved by TLC as described above, and visualized by autoradiography. No significant GTP hydrolysis by RhoA was observed during elution under these conditions.

Site-directed mutagenesis. The RhoE coding sequence was subcloned into the pSELECT vector (Promega), and site-directed mutagenesis was carried out with the pSELECT system according to the manufacturer's instructions with oligonucleotides which specifically alter codon 17, 64, or 66 (corresponding to Ras position 12, 59, or 61). The mutants were sequenced to confirm the predicted nucleotide changes and then subcloned into the pGEX vector. GST-RhoE mutant fusion proteins were expressed and assayed for GTPase activity as described above. Mutagenesis of the carboxy-terminal sequences of RhoE was conducted by PCR using synthetic oligonucleotides corresponding to the sequence of the 3′ end of the coding sequence that incorporate the relevant alterations.

Subcellular fractionation of transfected COS cells. COS cells were transfected and harvested as described above. Cells from three to five 10-cm-diameter tissue culture dishes were resuspended in 500 μl of hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 5 mM KCl, 10 μg of aprotonin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) and homogenized in a Dounce homogenizer with 100 strokes. Samples were then centrifuged at 3,000 rpm to pellet unlysed cells and cell debris. Part of the supernatant (homogenized total) was then subjected to ultracentrifugation at 100,000 × *g*, and the resulting supernatant was collected as the S100 fraction, while the pellet was the P100 fraction. The volumes of the homogenized total and the S100 and P100 fractions were adjusted so that equal percentages of starting material could be resolved by SDS-PAGE (15% gel). The HA-tagged proteins were then analyzed by immunoblotting with anti-HA antibody 12CA5 by using standard methods and detected by enhanced chemiluminescence, using Renaissance chemiluminescence reagent (DuPont NEN).

Metabolic labeling with [^{3}H]mevalonate in transfected COS cells. COS cells (3×10^6/100-mm-diameter dish) were cotransfected by the addition of 15 μg of plasmid Rc/CMV containing cDNA for either wild-type HA-RhoE, HA-RhoE-CTVL, or HA-RhoE-dCTVM (see Results) and 15 μg of pMEV (a mammalian expression vector containing the cDNA for a mevalonate transporter) in 3 ml of DMEM, using the DEAE-dextran method. At 32 h posttransfection, lovastatin was added to a final concentration of 20 μM and cells were incubated for 1 h at 37°C, whereupon the medium was aspirated and supplemented DMEM containing 20 μM lovastatin and 50 μCi of [5-^{3}H]mevalonate per ml was added. Following a 12-h incubation at 37°C, the labeling medium was removed, and the cells were washed with phosphate-buffered saline (PBS). The cells were released from the plate by scraping with a rubber policeman into PBS, pelleted by centrifugation, and flash frozen in liquid nitrogen. [5-^{3}H]mevalonolactone (60 Ci/mmol) was from American Radiolabelled Chemicals (St. Louis, Mo.); lovastatin was a generous gift from Al Alberts (Merck); pMEV was obtained from the American Type Culture Collection (Rockville, Md.); and trifluoroacetic acid and iodomethane were from Aldrich (Milwaukee, Wis.).

Isoprenoid analysis. Lysates from transfected COS cells were subjected to immunoprecipitation with anti-HA antibodies, and tagged RhoE proteins were precipitated with 15% trichloroacetic acid and washed extensively in acetone at −20°C. Trichloroacetic acid-precipitated proteins were digested with trypsin, and isoprenoids were cleaved from tryptic peptides by incubation with methyl iodide in 2% formic acid. Following cleavage, methyl iodide was removed from the solution under reduced pressure, and the solution was neutralized by addition of sodium carbonate. Isoprenoids were extracted into a solution of chloroform-methanol (9:1), and the extracted isoprenoids were dried under nitrogen and stored at −20°C. Immediately prior to analysis, samples were resuspended in 50% acetonitrile containing 0.1% phosphoric acid, and 16 μM each farnesol and geranylgeraniol were added to the samples as standards. Isoprenoids were resolved by C_{18} reverse-phase high-pressure liquid chromatography (HPLC), using a 50 to 100% linear gradient of acetonitrile in 0.1% phosphoric acid. Fractions containing ^{3}H-labeled isoprenoids were identified by liquid scintillation counting.

Immunofluorescent staining of U2OS cells. U2OS human osteosarcoma cells were plated at low density (30% confluence) and transiently transfected by the calcium phosphate method (20 μg of DNA per 10-cm-diameter dish). Sixteen hours posttransfection, cells were trypsinized and replated onto 15-mm-diameter round glass coverslips. Forty-eight hours posttransfection, cells were washed

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1                        24
CAGAAATTATCCAGCAAATCTATC

25/1                                    55/11
ATG GAT CCT AAT CAG AAC GTG AAA TGC AAG ATA GTT GTG GTG GGA GAC AGT CAG TGT GGA
Met asp pro asn gln asn val lys cys lys ile val val val gly asp ser gln cys gly
85/21                                   115/31
AAA ACT GCG CTG CTC CAT GTC TTC GCC AAG GAC TGC TTC CCC GAG AAT TAC GTT CCT ACA
lys thr ala leu leu his val phe ala lys asp cys phe pro glu asn tyr val pro thr
145/41                                  175/51
GTG TTT GAG AAT TAC ACG GCC AGT TTT GAA ATC GAC ACA CAA AGA ATA GAG TTG AGC CTG
val phe glu asn tyr thr ala ser phe glu ile asp thr gln arg ile glu leu ser leu
205/61                                  235/71
TGG GAC ACT TCG GGT TCT CCT TAC TAT GAC AAT GTC CGC CCC CTC TCT TAC CCT GAT TCG
trp asp thr ser gly ser pro tyr tyr asp asn val arg pro leu ser tyr pro asp ser
265/81                                  295/91
GAT GCT GTG CTG ATT TGC TTT GAC ATC AGT AGA CCA GAG ACC CTG GAC AGT GTC CTC AAA
asp ala val leu ile cys phe asp ile ser arg pro glu thr leu asp ser val leu lys
325/101                                 355/111
AAG TGG AAA GGT GAA ATC CAG GAA TTT TGT CCA AAT ACC AAA ATG CTC TTG GTC GGC TGC
lys trp lys gly glu ile gln glu phe cys pro asn thr lys met leu leu val gly cys
385/121                                 415/131
AAG TCT GAT CTG CGG ACA GAT GTT AGT ACA TTA GTA GAG CTC TCC AAT CAC AGG CAG ACG
lys ser asp leu arg thr asp val ser thr leu val glu leu ser asn his arg gln thr
445/141                                 475/151
CCA GTG TCC TAT GAC CAG GGG GCA AAT ATG GCC AAA CAG ATT GGA GCA GCT ACT TAT ATC
pro val ser tyr asp gln gly ala asn met ala lys gln ile gly ala ala thr tyr ile
505/161                                 535/171
GAA TGC TCA GCT TTA CAG TCG GAA AAT AGC GTC AGA GAC ATT TTT CAC GTT GCC ACC TTG
glu cys ser ala leu gln ser glu asn ser val arg asp ile phe his val ala thr leu
565/181                                 595/191
GCA TGT GTA AAT AAG ACA AAT AAA AAC GTT AAG CGG AAC AAA TCA CAG AGA GCC ACA AAG
ala cys val asn lys thr asn lys asn val lys arg asn lys ser gln arg ala thr lys
625/201                                 655/211
CGG ATT TCA CAC ATG CCT AGC AGA CCA GAA CTC TCG GCA GTT GCT ACG GAC TTA CGA AAG
arg ile ser his met pro ser arg pro glu leu ser ala val ala thr asp leu arg lys
685/221
GAC AAA GCG AAG AGC TGC ACT GTG ATG TGA
asp lys ala lys ser cys thr val met OPA

715
ATCTTTCATTATCTTTAATGAAGACAAAGGAATCTAGTGTAAAAAACAACAGCAAACAAAAAGGTGAGTCTAAATGAAGT
GCACAGCCAAAGTCATGTATACCAGAGGCTTAGGAGGCG  833
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FIG. 1. Human RhoE coding sequence. The nucleotide sequence of RhoE was determined from four independent cDNA clones isolated from a fetal human brain cDNA library. The predicted amino acid sequence (229 amino acids) is also shown. Only partial 5′ and 3′ untranslated sequences were determined. An in-frame stop codon was identified slightly further upstream, suggesting that the ATG codon indicated at amino acid position 1 is used to initiate translation (not shown).

briefly in PBS, then fixed for 20 min at room temperature in 3.7% formaldehyde in PBS, permeabilized for 5 min in 0.1% Triton X-100 (in PBS), and blocked for 5 min in 0.5% BSA (in PBS). Cells on each coverslip were then incubated with monoclonal antibody 12CA5 in PBS for 1 h at room temperature. After three washes in PBS (5 min each), cells were incubated with a 1:100 dilution of rhodamine-conjugated goat anti-mouse secondary antibody for 30 min at room temperature in PBS. Cells were then washed with PBS three times (5 min each), and coverslips were inverted and mounted on glass slides, using mounting solution containing 0.1% *p*-phenylenediamine antiquenching agent. Slides were then viewed on a Nikon Microphot fluorescence microscope. For confocal imaging, slides were viewed with a Bio-Rad MRC-600 confocal system. The antivinculin antibody was from Sigma.

RESULTS

Identification of cDNAs encoding a novel Rho family protein. p190 RhoGAP was first identified as the major binding partner of p120 RasGAP and was later found to be, itself, a GAP that specifically promotes the GTPase activity of several Rho family proteins (44). To identify additional cellular proteins that interact with p190 RhoGAP, a yeast interaction trap screen was performed with the complete p190 coding sequence as bait. Among the positive clones identified from a HeLa cell library was a cDNA clone that predicts strong sequence similarity to Rho family GTPases. As the clone appeared to represent a partial sequence, it was used as a probe to isolate complete cDNAs from a human fetal brain library. Four overlapping cDNA clones were isolated, and the complete coding sequence was assembled (Fig. 1). A single major open reading frame that exhibits sequence similarity to several members of the Rho GTPase family was identified (Fig. 2). The strongest homology is to the mammalian RhoA, -B, and -C proteins (54% identity), and weaker homology is seen to Rac1 and Rac2 (46% identity) and to CDC42 (43% identity). Similarity to Ras is also apparent (27% identity). Thus, this cDNA appears to represent a novel member of the Rho GTPase family of cytoskeletal regulatory proteins. We propose the name RhoE for this protein, as a distantly related protein, named RhoD, has been identified but not yet reported (18a). Although we have determined that the interaction of RhoE with p190 requires the p190 catalytic domain, thus far we have been unable to find evidence for a stable interaction between RhoE and p190 outside of the yeast system (unpublished results).

Northern blot analysis revealed that the RhoE gene is widely expressed as a relatively abundant 3.5-kb mRNA (Fig. 3). We have determined that the RhoE transcript contains approximately 1.5 kb each of 5′ and 3′ untranslated sequences (data not shown). The predicted RhoE protein has amino- and carboxy-terminal sequence extensions relative to previously described Rho proteins (Fig. 1). The amino terminus contains an additional 5 amino acids, and the carboxyl terminus contains an additional 65 amino acids. Thus, the predicted size of RhoE is approximately 26 kDa. Like other small GTPases, the car-

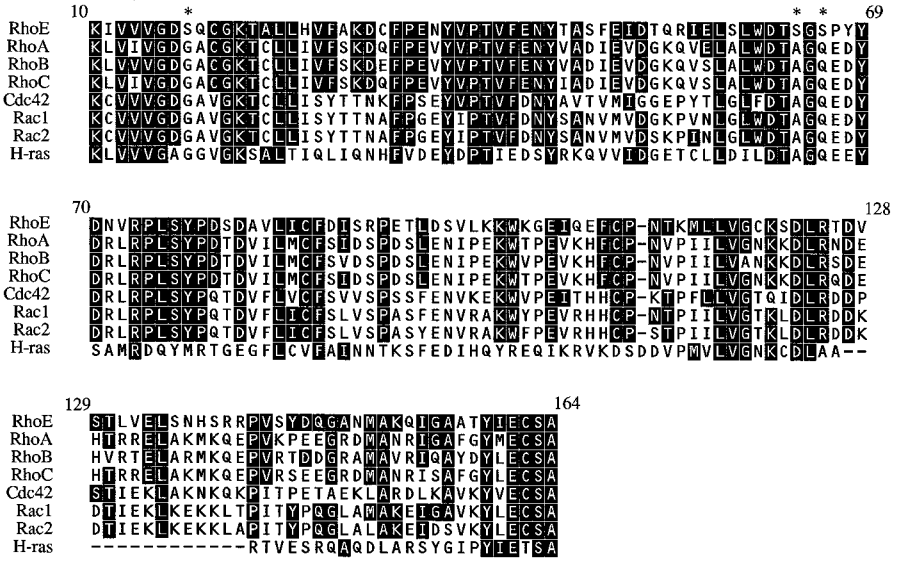

FIG. 2. Sequence alignment of RhoE and several small GTPases. A single major open reading frame that exhibits strong sequence similarity (43 to 53% identity) to several members of the Rho GTPase family was identified. Similarity to Ras is also apparent (27% identity). The predicted coding sequence of RhoE (single-letter code) was manually aligned with those of RhoA, RhoB, RhoC, Rac1, Rac2, CDC42, and H-Ras over a 155-amino-acid region that is highly conserved among all small GTPases. Letters in reverse font indicate amino acid identities between RhoE and at least one additional protein shown in the alignment. Asterisks highlight positions 17, 64, and 66 in RhoE, which correspond to residues 12, 59, and 61 in H-Ras.

boxy-terminal region is enriched in basic amino acids, although there is no polybasic domain as is seen in some Ras and Rho proteins. The carboxyl terminus of RhoE contains the sequence CTVM, which is predicted to undergo lipid modification, as has been found for other small GTPases. However, the presence of a carboxy-terminal methionine, which is also seen in K- and N-Ras, suggests that unlike other Rho proteins that are geranylgeranylated, RhoE might be modified by farnesylation.

The sequence of RhoE, which includes the conserved effector domain seen in other Rho family members, clearly places it in the Rho subfamily. However, RhoE has structural features that distinguish it from previously described Rho GTPases as well as from Ras, Rab, and Arf proteins. Most notably, RhoE has different amino acids at highly conserved positions that are known to be critical for normal GTP hydrolysis. Positions 17, 64, and 66 in RhoE, which correspond to amino acids 12, 59, and 61 in Ras, are substituted relative to most of the previously characterized small GTPases. At each position, a serine is found in the RhoE sequence. These three positions in Ras, when substituted with a variety of amino acids, confer oncogenicity by rendering Ras defective for GTP hydrolysis and resistant to GAP stimulation, thereby causing Ras to be maintained in an activated state (9, 11, 13, 22, 48). Substitutions at positions 14 and 63 in Rho proteins (analogous to positions 12 and 61) are also defective for GTPase activity and resistant to RhoGAPs, suggesting that the basic structural requirements for GTPase activity by Ras and Rho proteins are conserved (15, 44, 50–52).

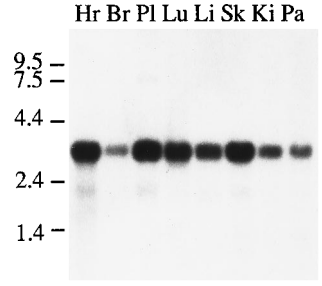

FIG. 3. Expression of the RhoE gene. A Northern blot (Clontech) containing RNA isolated from several human tissues was probed by hybridization to a full-length ^{32}P-labeled RhoE cDNA clone. Hybridization and washing were performed under standard high-stringency conditions. Hr, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; Sk, skin; Ki, kidney; Pa, pancreas. Sizes are indicated in kilobases.

RhoE binds GTP but lacks intrinsic GTPase activity. By analogy to mutationally activated Ras proteins, it was predicted that RhoE should exhibit a deficiency in GTPase activity. To test this possibility, a biochemical analysis of GTP binding and hydrolysis by RhoE expressed as a bacterial fusion protein was performed. Soluble RhoE fusion protein was expressed at high levels in bacteria and was easily purified on glutathione-agarose beads, as revealed by SDS-PAGE and Coomassie blue staining (see Fig. 5c). Like Ras and Rho GTPases, RhoE binds GTP rapidly upon incubation with radiolabeled nucleotide under standard nucleotide exchange conditions. Nucleotide binding is saturable and can be blocked by excess (1 mM) unlabeled GTP but not by ATP, CTP, or UTP, indicating that RhoE specifically associates with guanine nucleotide (data not shown). A nucleotide dissociation assay, in which the rate of release of radiolabeled GTP from preloaded protein was measured, revealed that RhoE has a considerably increased affinity for nucleotide relative to H-Ras and a moderately greater affinity than RhoA (Fig. 4a). As with Ras, nucleotide affinity of RhoE is reduced in low magnesium concentrations, although to a lesser extent than for Ras (19) (Fig. 4a). Thus, RhoE exhibits a specific, high-affinity binding to guanine nucleotide similar to that of other small GTPases.

We next assayed the ability of RhoE to hydrolyze GTP in a filter binding assay (Fig. 4b) and by TLC (Fig. 4c). Under conditions in which RhoA hydrolyzes more than 75% of bound

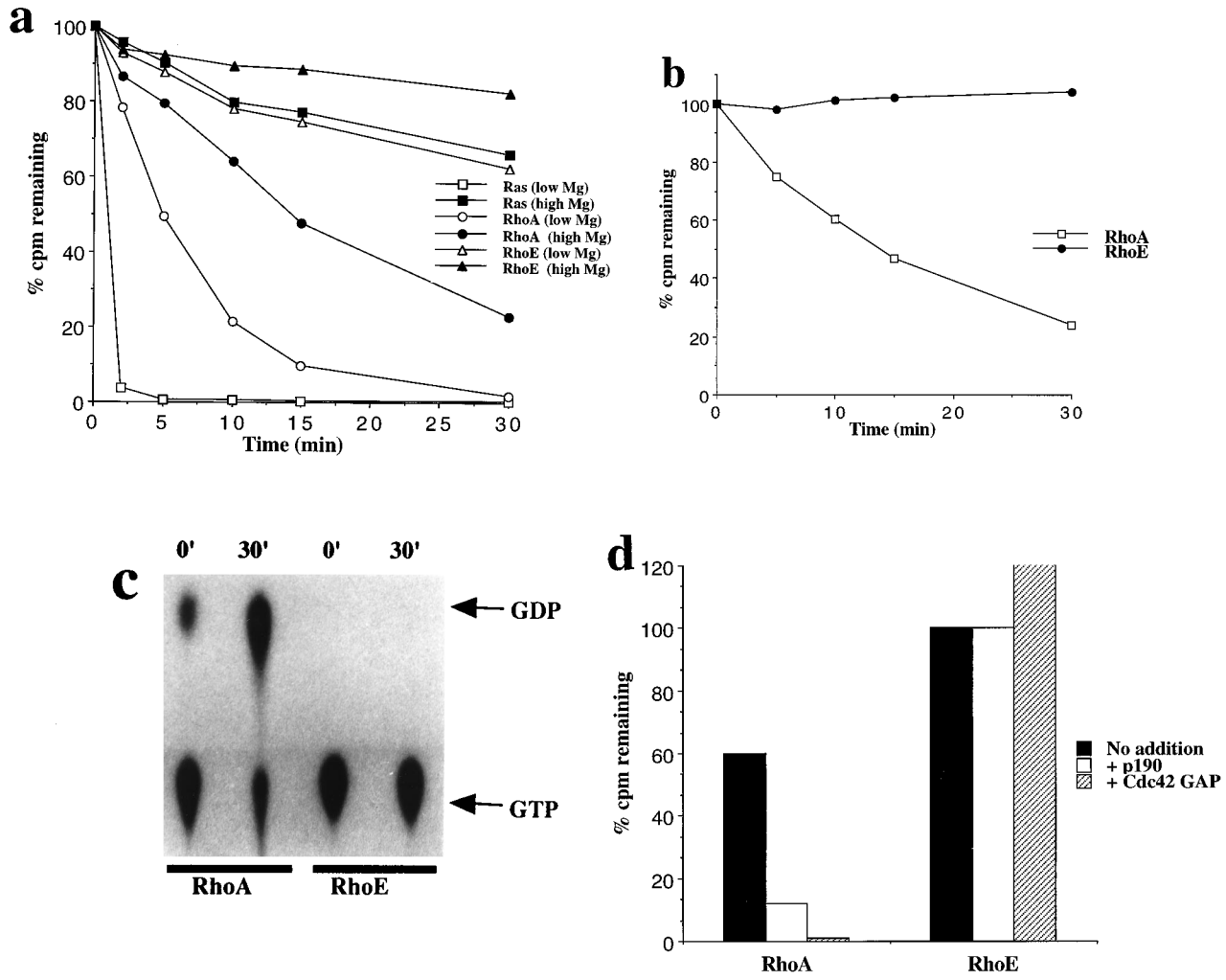

FIG. 4. RhoE binds GTP but lacks GTPase activity and is resistant to RhoGAPs. (a) Nucleotide dissociation assay of H-Ras, RhoA, and RhoE under conditions of high or low magnesium concentration. Purified, bacterially expressed fusion proteins were preloaded with [α-^{32}P]GTP, incubated at 37°C for the indicated times in the presence of excess (1 mM) unlabeled GTP, and subjected to a filter binding assay to quantitate the remaining radioactivity. (b) GTP hydrolysis by RhoA and RhoE in a filter binding assay. Each purified protein was preloaded with [γ-^{32}P]GTP, incubated at 37°C for the indicated times, and subjected to a filter binding assay to quantitate GTP hydrolysis. (c) GTP hydrolysis by RhoA and RhoE in a TLC assay. GST-RhoA and GST-RhoE fusion proteins bound to glutathione-agarose beads were preloaded with [α-^{32}P]GTP and incubated at 37°C for the indicated times (minutes), and eluted nucleotide was analyzed by TLC and autoradiography. The small amount of GDP seen in the RhoA sample at time zero reflects a low level of hydrolysis that occurs at 4°C prior to incubation at the assay temperature. (d) RhoGAPs stimulate RhoA but not RhoE. RhoA and RhoE fusion proteins were preloaded with [γ-^{32}P]GTP, incubated at 30°C in the presence or absence of p190 RhoGAP or CDC42 GAP for the indicated times, and subjected to a filter binding assay to quantitate GTP hydrolysis.

GTP, RhoE did not hydrolyze GTP detectably during a 30-min incubation at 37°C in either assay, suggesting that RhoE lacks intrinsic GTPase activity (<0.001 min^{-1}). Varying the pH and salt concentration or addition of detergents and lipids that promote hydrolysis by other small GTPases did not result in any detectable GTPase activity by RhoE, even after a 2-h incubation at 37°C (data not shown). Moreover, identically prepared samples of RhoB, H- and K-Ras, Rac1, CDC42, and Rap1 all exhibit GTPase activity under similar test conditions (data not shown). RhoE's inability to hydrolyze GTP is consistent with the amino acid differences between RhoE and other GTPases. A thrombin-cleaved version of RhoE that lacks the GST portion is also defective for GTP hydrolysis (not shown).

RhoE is resistant to RhoGAP activity. The down-regulation of small GTPases is achieved largely through their interaction with specific GAPs that strongly promote their intrinsic GTPase activities. Each of the Rho family proteins that have been characterized biochemically thus far is susceptible to the activity of at least one of several Rho-specific GAPs that have been identified (26). Since RhoE was identified by its interaction with p190 RhoGAP, we assayed the ability of p190 (a potent GAP for at least five Rho family members [44]) and another member of the RhoGAP family, CDC42 GAP (which stimulates Rho and CDC42 [4, 27]), to promote GTP hydrolysis by RhoE. Incubation of [^{32}P]GTP-loaded RhoE with purified preparations of p190 or CDC42 GAP failed to promote detectable GTP hydrolysis by RhoE under conditions in which RhoA activity was strongly promoted by both GAPs (Fig. 4d). Moreover, incubation of GTP-loaded RhoE with concentrated whole cell protein extracts from a variety of tissues (heart, lung, brain, liver, kidney, skeletal muscle, spleen, testes, and skin) also failed to promote GTP hydrolysis by RhoE (data not shown).

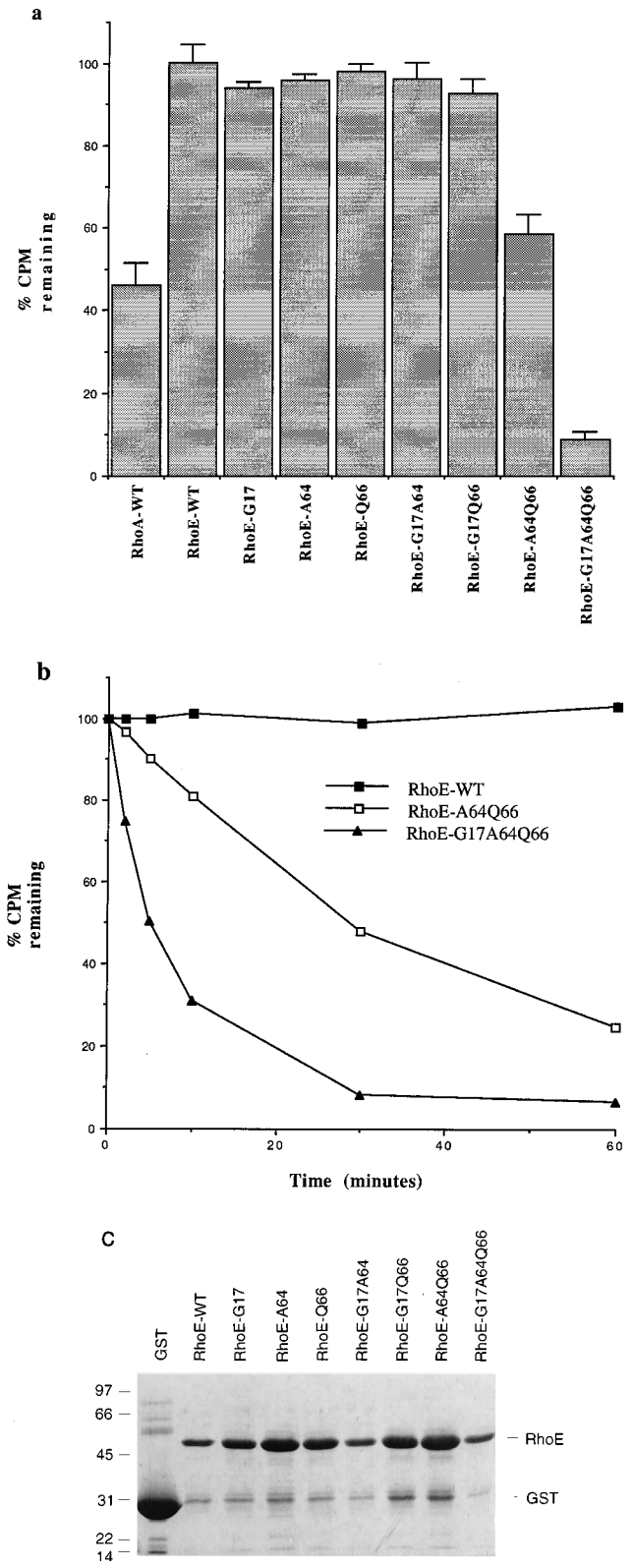

FIG. 5. Specific amino acid substitutions in RhoE restore GTPase activity. (a) Purified bacterial fusion proteins for wild-type RhoA (RhoA-WT), wild-type RhoE (RhoE-WT), and several specifically mutated forms of RhoE were loaded with [γ-^{32}P]GTP, incubated for 30 min at 30°C, and assayed for GTPase activity in a filter binding assay. The numbers for the various RhoE samples that were tested indicate amino acid positions that were substituted by site-directed mutagenesis. In each case, the indicated amino acid(s) was replaced with the corresponding amino acid(s) found in other Ras and Rho family proteins (as in Fig. 2). Each datum point represents the average of three independent experiments. (b) Kinetics of GTP hydrolysis by wild-type RhoE (RhoE-WT), RhoE-A64Q66, and RhoE-G17A64Q66. GTPase assays were performed as described above over the indicated time course for wild-type RhoE and the two mutants that exhibit GTPase activity. (c) Expression of wild-type and mutant RhoE as GST fusion proteins in bacteria. Each protein shown was purified on glutathione beads and analyzed by SDS-PAGE and Coomassie blue staining. Sizes are indicated in kilodaltons.

Specific amino acid substitutions in RhoE restore GTPase activity. To determine whether the amino acid differences in RhoE that correspond to oncogenic Ras mutations account for its lack of GTPase activity, we used site-directed mutagenesis to replace amino acids 17, 64, and 66 with their normal counterparts seen in other Ras and Rho proteins (Gly-12, Ala-59, and Gln-61, respectively). Each of these mutants was constructed individually and in all possible combinations and then expressed in bacteria (Fig. 5c) and assayed for GTPase activity. As shown (Fig. 5a), none of the three individual substitutions restore GTPase activity to RhoE. Two of the three double substitutions (G17A64 and G17Q66) also fail to restore GTPase activity. The A64Q66 double substitution, however, can hydrolyze GTP at levels comparable to those for RhoA, and the triple substitution (G17A64Q66) restores GTPase activity to a level substantially greater than that seen with RhoA (Fig. 5a and b). The GTPase-competent triple-mutant form of RhoE is still completely resistant to RhoGAP activity, indicating that other amino acid positions determine susceptibility to GAP activity (data not shown).

RhoE is constitutively associated with GTP in vivo. When the Ras protein is immunopurified from [^{32}P]phosphate-labeled cultured cells, it is found in both GTP-bound and GDP-bound states. Normally, the inactive GDP-bound form is predominant, and activation to the GTP form can be rapidly induced by a variety of mitogenic stimuli (42). On the basis of the observed absence of GTPase activity by RhoE and resistance to RhoGAPs in vitro, it was predicted that the GTP-bound form of RhoE would persist in vivo. To test this possibility, we examined the nucleotide state of RhoA and RhoE in [^{32}P]phosphate-labeled COS cells following transfection with mammalian expression vectors. Expression of the transfected constructs was confirmed by Western blotting (data not shown). Immunoprecipitation of each protein from ^{32}P-labeled lysates of transfected cells via an amino-terminal epitope tag and TLC of the eluted nucleotide revealed that while RhoA is associated with approximately equivalent amounts of GTP and GDP in vivo, RhoE is associated only with GTP (Fig. 6).

RhoE is farnesylated in vivo. In addition to having an unusual structure within the highly conserved catalytic domain, RhoE has a carboxy-terminal methionine, suggesting that it may normally be farnesylated (34, 39). Such a modification is not typically associated with the Rho family proteins (which are geranylgeranylated) and is generally characteristic of the Ras proteins, in which it is required for proper localization to the plasma membrane (16). Therefore, we examined the subcellular localization and prenylation of RhoE. For these experiments, cultured cells were transfected with plasmids encoding cytomegalovirus promoter-driven epitope-tagged forms of RhoE. To assess the role of prenylation in the subcellular localization of RhoE, two specifically mutated forms of the protein were expressed in addition to the wild-type protein. RhoE-CTVL contains a change of the carboxy-terminal methionine to leucine, resulting in a protein that is predicted to undergo geranylgeranylation instead of farnesylation. RhoE-

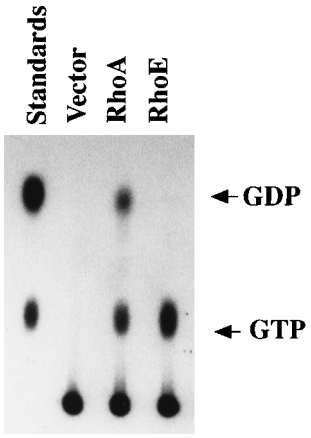

FIG. 6. RhoE is associated only with GTP in vivo. COS cells were transfected with a mammalian expression vector (Rc/CMV; Invitrogen) containing HA epitope-tagged versions of RhoA or RhoE (empty vector was transfected as a negative control). After 24 h, cells were metabolically labeled for 18 h with $^{32}P_i$, collected, lysed, and subjected to immunoprecipitation with an anti-HA antibody. After extensive washing of the immunoprecipitates, eluted nucleotide was subjected to TLC to resolve GTP and GDP. GTP and GDP standards were also run to indicate their migration.

dCTVM contains a deletion of the carboxy-terminal four amino acids of RhoE and is predicted to be defective for prenylation of either type. Wild-type RhoE and the two mutants were expressed transiently in transfected COS cells. All of the RhoE proteins were expressed at similar levels 48 h posttransfection, as revealed by anti-HA immunoblotting (Fig. 7).

To investigate the subcellular distribution of RhoE and the CAAX motif mutants, we transiently transfected RhoE expression vectors into COS cells, homogenized cells in hypotonic lysis buffer (48 h posttransfection), and fractionated the cellular proteins biochemically into a soluble S100 fraction and a particulate (presumably membrane-associated) P100 fraction by ultracentrifugation at 100,000 $\times$ *g*. Fractions were then subjected to immunoblot analysis. Approximately 60% of the wild-type RhoE distributes to the P100 fraction, while about 80% of the RhoE-CTVL mutant is in the P100 fraction (Fig. 8). In contrast, the vast majority of the RhoE-dCTVM mutant, whose CAAX motif is deleted, distributes to the S100 fraction. This experiment was repeated several times with the same results. The fractionation was judged to be essentially complete, since Coomassie blue staining of the same samples revealed that some protein bands were distributed completely to the S100 fraction while others were seen only in the P100 fraction (data not shown). Thus, these data suggest that RhoE

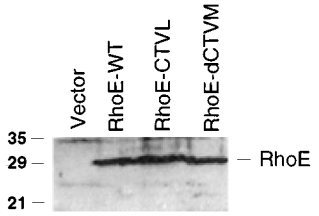

FIG. 7. Expression of RhoE proteins in transfected COS cells. Lysates from COS cells transiently transfected with plasmids expressing HA-tagged forms of wild-type RhoE (RhoE-WT) or the RhoE-CTVL and RhoE-dCTVM mutants were subjected to immunoblotting with an anti-HA antibody. Size markers are indicated in kilodaltons.

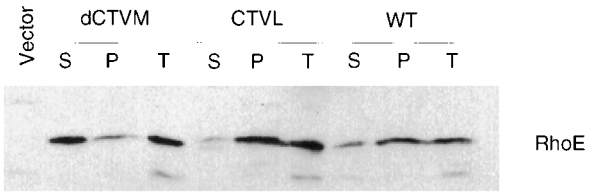

FIG. 8. Subcellular fractionation of RhoE. Lysates from COS cells transiently transfected with plasmids expressing HA-tagged forms of wild-type RhoE (WT) or the RhoE-CTVL and RhoE-dCTVM mutants were fractionated to P100 (P) and S100 (S) fractions as described in Materials and Methods and analyzed by immunoblotting with an anti-HA antibody. For comparison, total lysates (T) of corresponding transfections were analyzed similarly.

is probably prenylated and that this modification is important for determining RhoE's subcellular localization.

To test the possibility that RhoE is normally farnesylated, as predicted by its CAAX motif sequence, COS cells were transfected with the wild-type and mutated forms of RhoE and metabolically labeled with [^{3}H]mevalonate, a precursor for both farnesyl and geranylgeranyl groups. After immunoprecipitation of the HA-tagged RhoE protein, the associated radioactive prenyl groups were released and analyzed by reverse-phase HPLC together with C_{15} farnesyl group and C_{20} geranylgeranyl group standards (Fig. 9). As expected, wild-type RhoE is solely farnesylated, the RhoE-CTVL mutant is geranylgeranylated, and the RhoE-dCTVM mutant is not detectably prenylated.

To complement the biochemical fractionation results, we used immunofluorescence (with the anti-HA antibody) to explore further the subcellular localization of RhoE. Since the morphology of the transfected COS cells was not optimal, we chose to use the human osteosarcoma cell line U2OS, which maintains good morphology following transient transfection by the calcium phosphate method. As shown in Fig. 10A, wild-type RhoE expressed in transfected U2OS cells is seen diffusely in the cytoplasm, in association with plasma membrane, and in an unidentified cellular structure with a worm-like appearance. Notably, these worm-like structures are seen predominantly at the ventral surface of the cells, consistent with a role in adhesion. This is most clearly seen in confocal images of the most ventral focal plane (Fig. 10D). These structures do not resemble focal adhesions, which were revealed by immunostaining with an antivinculin antibody (Fig. 10E). The RhoE-CTVL mutant exhibits a similar staining pattern (Fig. 10B). The RhoE-dCTVM mutant fails to localize to the plasma membrane and instead is seen predominantly in the nucleus (Fig. 10C). Analogous experiments with nonprenylated Ras proteins revealed a similar nuclear accumulation of protein (20). In these experiments, transfected cells, which stain quite strongly, are easily seen among the nontransfected cells on the same slide by immunofluorescence with the anti-HA tag antibody. We cannot, however, formally rule out the possibility that the observed localization of RhoE is due to overexpression of the protein in transfected cells. A definitive localization will have to await the development of specific antisera that allow the detection of the endogenous protein.

DISCUSSION

In a yeast interaction trap screen to identify proteins that interact with p190 RhoGAP, we identified cDNA clones encoding a novel member of the Rho family of GTP-binding proteins. In light of the facts that p190 does not exhibit detectable GAP activity toward RhoE and that we have been unable

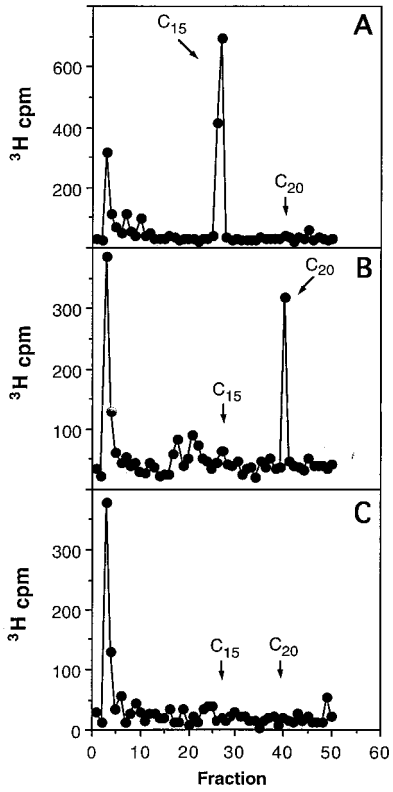

FIG. 9. Identification of the prenyl group attached to RhoE. COS cells expressing epitope-tagged versions of either wild-type RhoE (A), RhoE containing the C-terminal CAAX sequences CTVL (B), or HA-RhoE with a deleted CAAX (C) were labeled with [^{3}H]mevalonate. Proteins were immunoprecipitated with the anti-HA antibody, and the ^{3}H-labeled isoprenoids were cleaved from the proteins as described in Materials and Methods. Radioactivity extracted from the cleavage reactions was analyzed by reverse-phase HPLC as described in Materials and Methods. Elution of isoprenoid standards, marked by arrows, was monitored by A_{210} of authentic prenyl alcohols added to the samples prior to injection. C_{15}, *trans*,*trans*-farnesol; C_{20}, all-*trans*-geranylgeraniol.

to establish an interaction between these proteins outside of the yeast system, it is difficult to assess the physiologic relevance of the observed interaction between RhoE and p190. It is possible that the detection of this interaction simply reflects the ability of p190's RhoGAP domain to recognize a Rho-like structure in the highly sensitive yeast assay. As expected, the carboxy-terminal region of p190 that contains the RhoGAP domain is necessary for the p190-RhoE interaction (unpublished results). It remains possible, however, that the interaction is physiologically meaningful, with p190 potentially functioning as a downstream target of RhoE.

The finding that RhoE is farnesylated, while other Rho family proteins are geranylgeranylated, is intriguing and suggests that RhoE may exhibit unusual subcellular localization properties. The biological functions of small GTPases are mediated in part through their association with various cellular membranes. RhoE, like some of the other Rho family proteins (1), appears to distribute essentially equally between a soluble cytoplasmic form and a plasma membrane-associated form. In addition, immunostaining revealed that RhoE associates with cellular worm-like structures that we have yet to identify. The concentration of these structures at the ventral attachment surface of the cell suggests a possible role in adhesion. Indeed, other Rho proteins have recently been implicated in the regulation of adhesion in several systems (2, 32, 41, 47). The eventual identification of this putative adhesion structure will likely provide an important clue as to the precise biological function of RhoE. We have demonstrated that localization of RhoE to the plasma membrane as well as to this unknown structure requires prenylation of the carboxyl terminus. However, a geranylgeranylated form of RhoE appears to localize in a manner that is qualitatively indistinguishable from that of the wild-type farnesylated form of RhoE. The geranylgeranylated RhoE does, however, distribute preferentially to the particulate component in fractionation experiments. Thus, the unusual prenylation state of RhoE does not appear to provide RhoE with a particular subcellular localization that cannot be attained by geranylgeranylation of the protein. Similar results have been reported for a Ras mutant that was designed to undergo geranylgeranylation and which localizes normally to the plasma membrane and is biologically functional (8). Thus, the functional relevance of the distinct prenylation types for small GTPases remains unclear. It may be that the distinct lipid modifications of different small GTPases provide a regulatory mechanism that is too subtle to be detected by the methods described here. Notably, unlike the farnesylated Ras proteins, which are found exclusively at the plasma membrane, geranylgeranylated Rho proteins translocate between soluble and membrane-associated forms upon appropriate stimulation (47). Possibly, the prenylation difference influences the reversibility of the membrane interaction.

As has been found with Ras, RhoE protein that is not prenylated accumulates in the nucleus. This is most likely due to the presence of a cluster of basic amino acids found near the carboxyl termini of many small GTPases which may mimic a nuclear localization signal (20). However, the nuclear localization is not likely to be physiologically relevant. Notably, the prenylation-deficient form of RhoE fails to associate with the putative adhesion structure, indicating that this association also requires prenylation.

Interestingly, specific inhibitors of the farnesyltransferase enzyme, which have shown some success as antitumor drugs, have recently been found to revert Ras-transformed cells through a mechanism that does not appear to involve inhibition of Ras prenylation but instead appears to involve inhibition of an as yet unknown farnesyltransferase substrate that regulates the actin cytoskeleton (32). Since RhoE may be a cytoskeletal regulator that is farnesylated in vivo, it could be a target of these antitumor agents.

The primary structure of RhoE, which contains amino acid substitutions at three highly conserved positions that are important for normal GTPase activity by other Rho and Ras proteins, is somewhat unique. While a few small GTPases that contain substitutions at one or two of these positions have been identified, those proteins for which a biochemical analysis has been performed have been found to exhibit GTPase activity despite these differences (30, 40). For example, the Rap proteins contain a threonine at position 61, which is a glutamine in nearly all other mammalian GTP-binding proteins, and is able to hydrolyze GTP efficiently (21). In addition, the Rad and Gem proteins, which define a novel class of Ras-related (25% identity) GTP-binding proteins, have an unusual structure in some of the well-conserved nucleotide-binding motifs, and Rad has been found to exhibit GTPase activity and to be susceptible to a cellular GAP activity (53). Therefore, it appears that some deviation from the consensus structure within the catalytic region can be tolerated. However, at least for several Ras and Rho proteins that have been examined, substitutions at position 12 or 61 substantially impair catalytic function (9, 10, 15, 52). Moreover, substitutions at all three of these positions in any one GTPase are unprecedented.

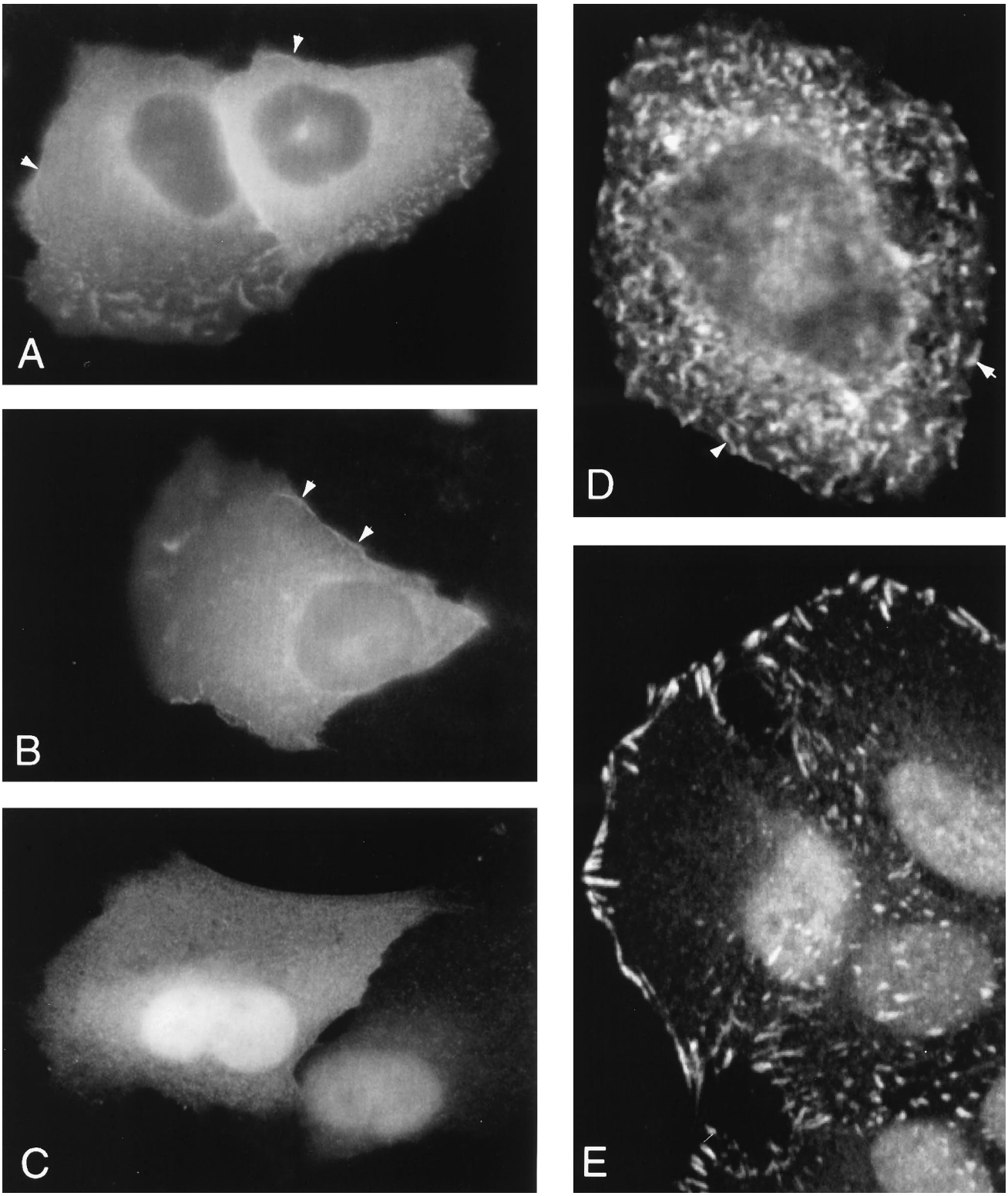

FIG. 10. Immunolocalization of RhoE in transfected U2OS cells. U2OS cells were transfected with HA-tagged forms of RhoE and analyzed by immunofluorescence with an anti-HA antibody. (A) Wild-type RhoE; (B) RhoE-CTVL; (C) RhoE-dCTVM. Arrows indicate plasma membrane staining. Confocal imaging of the ventral attachment plane of wild-type RhoE-transfected cells (D) reveals localization of RhoE to worm-like structures (arrowheads). Confocal imaging of U2OS cells immunostained with an antivinculin antibody reveals the appearance of focal adhesions in these cells (E).

Consistent with the biochemical properties of mutationally activated Ras proteins that contain amino acid substitutions within the catalytic site, RhoE exhibits no trace of intrinsic GTPase activity. Moreover, we have not been able to detect a GAP activity for RhoE, raising the possibility that cellular RhoE is constitutively in a GTP-bound state. Consistent with this possibility is our finding that RhoE expressed in transfected COS cells is found exclusively in a GTP-bound form. While it is possible that the nucleotide state of RhoE is not regulated normally when RhoE is overexpressed in COS cells as a result of limiting concentrations of some cellular GAP, the observations that the nucleotide state of transfected RhoA appears to be regulated and that we cannot detect a GAP activity for RhoE in highly concentrated cell extracts make this unlikely. It is not possible, however, to rule out formally the possibility that there is some physiological context in which RhoE is stimulated to hydrolyze GTP. Previously, the Arf proteins, which regulate vesicular trafficking, have also been reported to lack an intrinsic GTPase activity. However, unlike for RhoE, evidence indicates that these proteins cycle between GDP- and GTP-bound states in vivo, and an Arf-specific GAP has recently been identified (38).

The finding that replacement of the three substituted positions in RhoE with conventional amino acids imparts potent GTPase activity upon RhoE suggests that the three-dimensional structure of RhoE is probably quite similar to that of other small GTPases, with the exception of subtle differences in the nucleotide-binding site that severely compromise the ability to hydrolyze GTP. Interestingly, the murine sarcoma retrovirus Ras oncogenes encode a Ras protein which is mutationally substituted at both positions 12 and 59 (12, 31, 49). In this form of Ras, the defect in GTPase activity is more severe than that seen when the two substitutions are introduced individually, suggesting an additive activating effect of such substitutions in the catalytic site (22). This observation, together with the results of the mutational analysis of the putative catalytic domain of RhoE, suggests that the multiple substitutions in RhoE relative to other small GTPases account for its severely impaired ability to hydrolyze GTP. Thus, as for Ras, all three of these positions are important for hydrolytic function, and furthermore, they appear to cooperate to establish a functional catalytic site. The fact that all three positions are altered in RhoE while they have been so highly conserved in such a wide variety of GTPases throughout evolution (7) suggests that strong selective pressure was necessary to yield a protein in which the GTP-bound state is so predominantly favored. Notably, the selective pressure on Ras and on Gα subunits during the tumorigenesis process results in the same types of substitutions (5, 28, 29, 33, 46). The fact that the overall Rho-like structure of RhoE, including the putative effector domain (35), has been conserved suggests that, like other Rho family members such as RhoA, Rac, and CDC42 (18, 36), RhoE is likely to play some role in regulating the actin cytoskeleton. However, thus far, we have been unable to demonstrate a cytoskeletal effect of overexpressing RhoE, as has been found for other Rho family proteins (unpublished results).

By analogy to previously characterized GTP-binding proteins, it is expected that the GTP-bound state of RhoE is the active state (7, 24). If this is so, it is possible that RhoE activity is regulated by means other than nucleotide cycling. For example, RhoE expression levels, subcellular localization, or phosphorylation state (if it is phosphorylated) might determine the level of activity. In these cases, it is conceivable that the association with GTP plays only a structural role, perhaps coordinating the correct conformation of the protein. As such a role for GTP binding is unprecedented, we are hesitant to conclude that this mechanism applies to RhoE. In any case, it appears that RhoE is an unusual G protein that may normally be maintained in a GTP-charged state and may not be regulated by a conventional nucleotide cycling mechanism. While studies of Ras and heterotrimeric G proteins have led to the conclusion that GDP release from inactive protein is the rate-limiting step in activation, the results of the RhoE analysis raise the possibility that nucleotide exchange may not be the universal activating mechanism for GTP-binding proteins.

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