Transient Palmitoylation Supports H-Ras Membrane Binding but Only Partial Biological Activity†

Sarah G. Coats,‡ Michelle A. Booden, and Janice E. Buss*

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

Received April 23, 1999; Revised Manuscript Received July 29, 1999

ABSTRACT: H-Ras is >95% membrane-bound when modified by farnesyl and palmitate, but <10% membrane-bound if only farnesyl is present, implying that palmitate provides major support for membrane interaction. However the direct contribution of palmitate to H-Ras membrane interaction or the extent of its cooperation with farnesyl is unknown, because in the native protein the isoprenoid must be present before palmitate can be attached. To examine if palmitates can maintain H-Ras membrane association despite multiple cycles of turnover, a nonfarnesylated H-Ras(Cys186Ser) was constructed, with an N-terminal palmitoylation signal, derived from the GAP-43 protein. Although 40% of the GAP43:Ras-(61Leu,186Ser) protein (G43:Ras61L) partitioned with membranes, the chimera had less than 10% of the transforming activity of fully lipidated H-Ras(61Leu) in NIH 3T3 cells. Poor focus formation was not due to incorrect targeting or gross structural changes, because G43:Ras61L localized specifically to plasma membranes and triggered differentiation of PC12 cells as potently as native H-Ras61L. Proteolytic digestion indicated that in G43:Ras61L both the N-terminal and the two remaining C-terminal cysteines of G43:Ras61L were palmitoylated. A mutant lacking all three C-terminal Cys residues had decreased membrane binding and differentiating activity. Therefore, even with correct targeting and palmitates at the C-terminus, G43:Ras61L was only partially active. These results indicate that although farnesyl and palmitate share responsibility for H-Ras membrane binding, each lipid also has distinct functions. Farnesyl may be important for signaling, especially transformation, while palmitates may provide potentially dynamic regulation of membrane binding.

Membrane binding of H-Ras is accomplished through a series of posttranslational modifications to its carboxy terminus: covalent attachment of the C15 isoprenoid farnesyl (*1*−*4*), proteolytic removal of the three C-terminal residues, methylation of the farnesylated cysteine, and palmitoylation of two nearby cysteines. The two types of lipid modifications differ significantly in the stability of their attachment. Farnesyl becomes attached permanently to H-Ras at Cys186 (*2*). Palmitate can be added to H-Ras at two sites, Cys181 and Cys184 (*3, 5*), but, in contrast to farnesyl, is attached reversibly, undergoing cycles of removal and addition with a half-time of 1−2 h (*6, 7*).

Membrane binding of H-Ras has received a great deal of attention because it is a property that is critical for its ability to function in intracellular signaling (*8*−*10*). However, the individual contributions of farnesyl and palmitate or the extent of their cooperation in membrane attachment has not been clarified. Farnesylation occurs first, and initiates membrane localization, and thus is in temporal control of this event. The role of palmitate in membrane association of H-Ras is not well-defined because the prerequisite of farnesylation has forced existing models to be built largely from mutants which lack this lipid. When modified by both farnesyl and palmitate, H-Ras is >95% membrane bound and fully active; with farnesyl as the only lipid, <10% of the H-Ras is attached to membranes, and biological activity is severely compromised (*11*−*14*). This shows that the farnesyl group by itself can support only a minimal amount of H-Ras membrane association and implies that palmitates are needed for full plasma membrane interaction. However, because such mutants show decreases in both membrane interaction and function, these results cannot answer whether palmitate or farnesyl make distinct contributions or share responsibility for both events.

An additional difficulty for studies of palmitoylation is the chemical lability of the palmitoyl−cysteine thioester linkage (*5*), which has prevented a direct assessment of the stoichiometry of H-Ras palmitoylation. Although H-Ras has two cysteines that can be palmitoylated, it is still not known what portion of these sites are occupied, or need to be, to attain the >95% membrane binding seen at steady state. In vitro measurements with fully dipalmitoylated peptides have shown that two acyl groups enable peptides to bind strongly to artificial membranes (*15*). However, in a biological setting it is unclear if the dynamic turnover of the palmitates might limit or set a threshold on the ability of the acyl groups to maintain membrane tethering of H-Ras.

† These studies were supported by USPHS Grant CA 51890, by the Roy J. Carver Charitable Trust, and by the Iowa Agriculture and Home Economics Experiment Station. This manuscript is journal paper 17257 and was supported by Hatch Act and State of Iowa funds.

* To whom correspondence should be addressed at 3212 Molecular Biology Building, Iowa State University, Ames, IA 50011. Phone: 515-294-6125. FAX: 515-294-0453. E-mail: jbuss@iastate.edu.

‡ Current address: Harvard Medical School, Boston, MA 02115-5750.

10.1021/bi9909290 CCC: $18.00
Published on Web 09/09/1999

We therefore sought ways to clarify to what extent transiently attached palmitates can, by themselves, maintain H-Ras interactions with membranes, and whether H-Ras needs both palmitates and a permanently attached farnesyl to achieve a biologically functional level of association.

A nonfarnesylated mutant of H-Ras (ExtRas) that is palmitoylated at the original C-terminal sites has previously been designed (*16*). ExtRas has six lysines added to its C-terminus, that are designed to initiate membrane interaction via ionic forces and subsequently enable the protein to be palmitoylated. Approximately 40% of ExtRas binds to membranes, and the protein has transforming activity equivalent to H-Ras61L with the native lipid modifications. Surprisingly, in both cellular and activated 61Leu forms, ExtRas causes an unusual and exaggerated differentiation of PC12 cells (*16*), and the effector protein, phosphatidylinositol 3′-kinase, binds ExtRas(61Leu) roughly 6-fold more effectively than H-Ras 61L with the full native lipid modifications (M.A.B. and J.E.B., manuscript in preparation). This combination of C-terminal palmitates and lysines thus appears to alter signaling in addition to supporting membrane association.

Therefore, a palmitoylated H-Ras protein with a C-terminus that was unaltered, except for the lack of a farnesyl, was desired, to maintain as nearly as possible the conformation and interactions of this domain. For this purpose, a chimeric Ras protein was created, by fusing a leader sequence, derived from the GAP-43 protein (*17*), to the amino terminus of H-Ras(Cys186Ser). GAP-43, also known as B-50 or neuromodulin, is palmitoylated at its amino terminus, on Cys3 and Cys4 (*18, 19*). Attachment of the N-terminal 11 residues of GAP-43 has been shown to enable a soluble protein to become palmitoylated and membrane-bound (*17, 18, 20*). Importantly, palmitoylation of GAP-43 is a dynamic modification, with a turnover rate of the GAP-43 palmitates that is similar to those of H-Ras (*18*).

In G43:Ras61L both the introduced N-terminal and the retained, native C-terminal cysteines appeared to be palmitoylated. This provided an opportunity to examine membrane binding and biological activity of a G43:Ras61L protein with a C-terminus whose only change was a specific lack of isoprenoid. The results indicate that, in the setting of the intact cell, dynamic acylation of both N- and C-terminii can support significant H-Ras membrane binding and one type of biological activity (differentiation). However, a second type of biological outcome, transformation, appeared to be particularly sensitive to the absence of farnesyl and the palmitate-dependent balance of membrane binding.

MATERIALS AND METHODS

Construction of Chimeric G43:Ras61L Genes. The DNA for a protein termed $G43_{11}61L$ was constructed first, using a small oligonucleotide designed to encode the first 11 amino acids of neuronal growth cone protein GAP-43, and the first 5 amino acids of H-Ras. The human H-Ras gene, from the *Hin*dIII site at codon 5 through the 3′ noncoding region, containing a Cys186Ser mutation (to prevent prenylation of Ras) and an activating Gln61Leu mutation (61L) was excised from a donor vector by simultaneous digestion with *Hin*dIII and *Bam*HI restriction endonucleases. The pcDNA3 vector (InVitrogen) was digested at the unique *Bam*HI site in the Multiple Cloning Region, and the three components (vector, oligonucleotide, and H-Ras) were joined by ligation (*21*). The presence of the correct nucleotide sequence in the final clones selected was confirmed by DNA sequencing. Polymerase chain reaction (PCR) was then used to create $G43_{10}$-61L, a new chimeric gene identical to the first with the exception that methionine 1 of H-Ras was deleted (Figure 1). The original $G43_{11}61L$ constructs were used as templates for the reaction, and primers were designed to encode the first 11 codons of GAP-43, and codons 2−5 of H-Ras as well as a *Bam*HI restriction site at each end. The chimeric protein which lacked all three C-terminal cysteines, $G43_{10}$-61L(*3Ser*), was constructed by PCR using the $G43_{10}61L$ DNA as template and a mutagenic oligonucleotide for the 3′ end of the DNA. The protein in which the N-terminal cysteines were replaced by serines, $G43_{10}61L$(*C3,4S*), was constructed in a similar fashion by PCR, using $G43_{10}61L$ DNA as template and a mutagenic oligonucleotide for the 5′ end of the DNA.

Focus Formation Assay. The G43:Ras61L genes were excised by *Bam*HI digestion and cloned into the mammalian retrovirus expression vector pZIP-neoSV(x)1 for use in NIH 3T3 cell transfection assays. DNA suitable for mammalian cell transfections was prepared using a Qiagen tip-100 column (Qiagen). NIH 3T3 mouse fibroblasts were plated at a density of 5×10^5 cells per 60 mm dish and transfected the following day with 0.1−10 μg of the appropriate plasmid DNA using calcium phosphate-mediated transfection (*22*). Cells were treated with trypsin the following day and divided equally into four dishes: three for focus analysis and one for G418 selection. Cells on focus plates were grown in DMEM + 10% calf serum; those on G418 selection plates were grown in DMEM + 10% calf serum + 400 μg/mL G418 (Genetecin, Gibco-BRL). Foci were counted at approximately 2 weeks, and the number of foci per microgram of DNA and the number of foci per 1000 G418 colonies calculated.

Soft Agar Assay. NIH 3T3 cells stably expressing chimeric or control proteins were seeded at a density of 10^4 single cells per 60 mm dish in a soft agar overlay of 0.4% Bacto-agar (Difco) in DMEM + 10% calf serum on top of a feeder layer of 0.6% Bacto-agar in the same medium. Cells were photographed after 2 weeks. The G418-selected v-H-Ras-expressing positive control NIH 3T3 cells were kindly provided by B. Willumsen, Copenhagen (*14*).

COS-1 and PC12 Cell Transfection. Liposome-mediated transfection was performed using Lipofectamine (Gibco-BRL). COS-1 cells were seeded at approximately 50% confluence 1 day prior to transfection, and then treated with 100 ng of the chimeric or control gene in a pcDNA3 vector. PC12 cells were obtained as a kind gift from Dr. J. H. P. Skene, Duke University, and were seeded at approximately 25% confluence 1 day prior to transfection and then treated with 1−3 μg of the chimeric or control gene in a pcDNA3 vector, using Lipofectamine (*16*).

Immunofluorescence. COS-1 cells that expressed chimeric or control proteins were plated at low density on serum-coated coverslips in 6-well culture dishes. Following fixation with 2% paraformaldehyde, cells were treated with 5% nonimmune rabbit serum to block nonspecific antibody interactions and 0.02% saponin to permeabilize the cells. Cells were then treated with a 1:20 dilution of Y13-172 rat

monoclonal antibody specific to H-Ras followed by a 1:50 dilution of FITC-conjugated rabbit anti-rat secondary antibody (Cappel/Organon-Teknicon), both diluted in blocking solution, mounted in DABCO−glycerol solution to prevent fading, and viewed by confocal immunofluorescence microscopy. PC12 cells expressing chimeric or control protein were treated similarly, except that the blocking solution used contained 0.4% bovine serum albumin, 3% horse serum, and contained 0.05% Triton X-100 and 0.05% Tween-20 instead of saponin as permeabilization agents (*19*).

Fractionation. Subcellular fractions were prepared by hypotonic lysis followed by ultracentrifugation at 100000*g* as previously described (*23*). Repeated suspension and resedimenting of the P-100 fractions with hypotonic buffer showed that G43:Ras protein remained associated with the membranes. For salt treatment of the membranes, the P-100 fraction was resuspended for 30 min on ice in hypotonic lysis buffer to which NaCl was added to achieve a concentration of 0.5 M. Separated fractions were treated with 4 volumes of acetone at 0 °C and precipitated proteins collected by centrifugation for analysis of total proteins; otherwise, fractions were reconstituted in "high-SDS RIPA buffer" to final concentrations of 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM sodium chloride, 1% (v/v) Aprotinin (Calbiochem) for immunoprecipitation. For testing detergent releasability of proteins from the P-100 membrane fraction, the supernatant (S100 = S1) was set aside, and the pellet from the 100000*g* spin was resuspended in 900 μL of TESV (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate, 1 μg/mL leupeptin, 1 mM pepstatin, 1 mM Pefabloc) and 100 μL of 10% NP-40 was added. The suspension was centrifuged at 16000*g* for 15 min and the NP-40 extract (S2) removed. The pellet (P) was resuspended in 100 μL of electrophoresis sample buffer. The S1 and S2 supernatants were treated with 10 mL of ice-cold acetone for 1 h and centrifuged at 3000 rpm for 30 min, and the precipitated proteins were dissolved in 100 μL of electrophoresis sample buffer.

Radiolabeling and Immunoprecipitation. G418-selected NIH 3T3 cells or lipofectamine-transfected COS-1 cells expressing chimeric or control H-Ras proteins were labeled 4 h with [^{3}H]palmitic acid (Dupont−NEN) at 1 mCi/mL and 50 μg/mL cyclohexmide, or overnight with 5-[^{3}H]mevalonolactone (American Radiolabeled Chemicals) at 200 μCi/mL, in the presence of 100 μM compactin to inhibit mevalonate biosynthesis (*24*). After the labeling period, subcellular fractions were prepared as described above, or cells were lysed directly in high-SDS RIPA buffer. Immunoprecipitation was performed with 3E4-146 mouse anti-H-Ras monoclonal antibody (Quality Biotech). All samples were resolved by SDS−PAGE and transferred to a PVDF membrane and, as needed, sprayed with En^{3}Hance (DuPont/NEN) for fluorography or developed with antibody for Western blot analysis.

Immunoblotting. Proteins collected by centrifugation or immunoprecipitation were resuspended in electrophoresis sample buffer (2% SDS, 10 mM sodium phosphate, pH 7.0, 10% glycerol, 5% β-mercaptoethanol, 100 mM DTT, 0.01% bromophenol blue). After separation by SDS−PAGE, proteins were transferred electrophoretically to a PVDF membrane, and nonspecific protein binding was blocked by incubating the membrane overnight in 2.5% nonfat dry milk in Tris-buffered saline. Membranes were probed with primary antibody 3E4-146 mouse anti-H-Ras, followed by Vectastain secondary antibody, alkaline phosphatase−enzyme complex, and Vectastain substrate (Vector Labs).

N-terminus of G43$_{10}$ constructs

GAP-43 (1–11) H-Ras (2–)

Met-Val-*Cys*-*Cys*-Met-Arg-Arg-Thr-Lys-Gln-Val-Thr-Glu-Tyr..

GATCCA CCATG GTG TGC TGT ATG AGA AGA ACC AAA CAG GTT ACA GAA TAC A

C-terminus of G43$_{10}$61L(*3Ser*)

H-Ras 181 184 186

-Asp-Glu-Ser-Gly-*Ser*-Met-Ser-*Ser*-Lys-*Ser*-Val-Leu-Ser (stop)

FIGURE 1: N- and C-terminal amino acids and oligonucleotides used to construct G43$_{10}$61L and G43$_{10}$61L(*3Ser*) chimeras. GAP-43 amino acids 1−11 (underlined) are fused to H-Ras amino acids 2−189. The two N-terminal cysteine residues available for palmitoylation are italicized. In the C-terminus of G43$_{10}$61L(*3Ser*), the three serines which replace the C-terminal cysteines that are normally lipidated are also italicized.

Proteolytic Cleavage. The protocol was modified from one published previously (*5*). COS-1 cells expressing H-Ras61L or G43$_{10}$61L were labeled with [^{3}H]palmitic acid and immunoprecipitates formed as described above. The samples were loaded into the wells of a 24% acrylamide SDS−PAGE gel and overlaid with 0.1, 1.0, or 10 μg of endoprotease Glu-C (*Staphylococcus aureus* strain V8; Sigma) dissolved in a nonreducing electrophoresis sample buffer (same as above except only 50 mM DTT and no β-mercaptoethanol). Electrophoresis was started and continued until the sample dye just entered the separating gel; then the current was stopped and proteolysis allowed to proceed for 30 min at ambient temperature. Electrophoresis was continued in order to resolve the resulting fragments on the 24% acrylamide separating gel; then proteins were transferred to a PVDF membrane and labeled proteins detected by fluorography as described above.

RESULTS

Construction and Expression of G43:Ras61L Chimeras. A number of nonfarnesylated chimeric H-Ras proteins with an alternative lipid modification (myristoylation) or transmembrane domain have been studied (*11*, *14*, *23*, *25*), but in each of these chimeric proteins the surrogate membrane targeting motif has been, like the farnesyl group, a permanent part of the H-Ras structure. To create an H-Ras in which palmitates were the only lipids and means of membrane attachment, a chimeric G43:Ras61L gene was designed, using the amino-terminal sequence of GAP-43 as a palmitoylation signal. The first 11 codons of GAP-43 were attached as a leader to codons 2−189 of an activated H-Ras-(61Leu/Cys186Ser) in which the 186Ser mutation prevented attachment of farnesyl (Figure 1). This protein in effect only lengthened the amino terminus of H-Ras by 10 residues, and was therefore termed G43$_{10}$61L. Immunoblots of cells transfected with the G43$_{10}$61L construct showed the 22 kDa band expected for the fusion protein (see Figure 3A). Another DNA containing the 11 codons of GAP-43 attached to the full-length 189 codons of H-Ras, termed G43$_{11}$61L, was also constructed. However, when the G43$_{11}$61L chimera was expressed in NIH 3T3 cells, although 70% of the protein was the size expected for the chimera, ~30% of the protein appeared to lack the GAP-43 leader sequence as a result of occasional initiation of translation at the retained Met1 of H-Ras, producing a 21 kDa protein that was found only in cytosolic fractions and, as previously shown for H-Ras186Ser

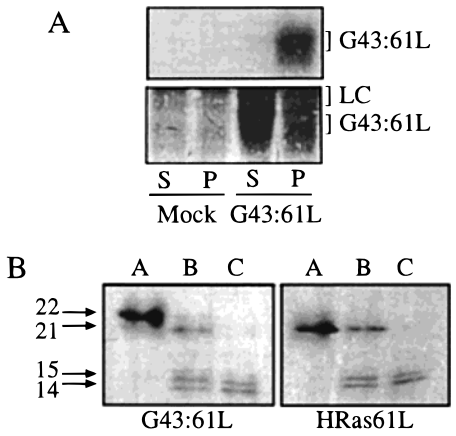

FIGURE 2: (A) G431061L is palmitoylated. COS-1 cells were transfected with either an empty pcDNA3 vector or the vector encoding $G43_{10}61L$, and 48 h later were labeled for 4 h with [^{3}H]-palmitic acid and lysed, and soluble, S-100 (S), and membrane-containing, P-100 (P), fractions were prepared. H-Ras protein was isolated by immunoprecipitation and resolved by SDS−PAGE for fluorographic exposure (exposure time 30 days; top panel). The membrane used to expose the film was then developed with H-Ras-specific antibody (bottom panel). Antibody light chains from the immunoprecipitate are indicated as LC. No [^{3}H]palmitate-labeled protein was observed in cells transfected with vector only, indicating that the band observed in the upper panel is a result of the introduced plasmid rather than endogenous H-Ras protein. (B) Proteolytic digestion indicates that $G43_{10}61L$ is palmitoylated at its carboxy terminus. COS-1 cells expressing H-Ras61L or $G43_{10}61L$ proteins were labeled with [^{3}H]palmitate for 4 h and H-Ras proteins isolated by immunoprecipitation. The samples were loaded in the wells of a 24% SDS−polyacrylamide gel and overlaid with 0.1 μg (lanes A), 1 μg (lanes B), or 10 μg (lanes C) of *Staphylococcus aureus* V8 endoprotease and processed as described under Materials and Methods. Protein fragments were transferred to a PVDF membrane and exposed to film for 60 days. Molecular weights of the [^{3}H]-palmitate-labeled peptides are indicated.

proteins, was not palmitoylated (data not shown). This characteristic of H-Ras fusion proteins had been observed previously for other chimeric H-Ras proteins (*23*). The chimeric forms of these two proteins exhibited similar biochemical (C-terminal palmitoylation) and biological behavior (poor NIH 3T3 cell focus formation and good PC12 cell differentiation), and results given here apply to both $G43_{10}$ and $G43_{11}$ forms. Two additional chimeric DNAs were also constructed. One, $G43_{10}61L$(*3Ser*), had codons 181, 184, and 186 for all three of the C-terminal cysteines replaced with nucleotides encoding serine residues, and was designed to produce a G43:Ras61L protein that had palmitates only at the N-terminus and lacked all C-terminal cysteines and their lipids (see Figure 1). The other, $G43_{10}61L$(*C3,4S*), replaced the N-terminal cysteines with serines, but retained the GAP-43 leader. The C-terminus of the $G43_{10}61L$(*C3,-4S*) protein had the 186Ser mutation to prevent farnesylation, but preserved the cysteines at positions 181 and 184.

G43:Ras61L Is Palmitoylated but Not Prenylated. To determine directly if the chimeric protein possessed the desired palmitate modification, COS-1 cells expressing $G43_{10}61L$ were labeled with [^{3}H]palmitic acid, and the chimeric Ras protein was isolated by immunoprecipitation. Figure 2A shows that the protein did incorporate palmitate, and that 100% of the palmitoylated protein partitioned into the P-100 fraction. After fluorographic exposure, the membrane was developed with H-Ras-specific antibody to determine the distribution of chimera between S-100 and P-100 fractions. The immunoblot showed that approximately 60% of the chimeric protein was soluble and 40% was present in the P-100 fraction. Because no [^{3}H]palmitate was present in the S100 fraction, the 60% of the protein that was cytosolic therefore was not palmitoylated. The palmitates that were present in the membrane-bound fraction of $G43_{10}61L$ underwent turnover, with a half-life similar to the palmitates present in native H-Ras (data not shown).

Cells expressing $G43_{10}61L$ or v-H-Ras were also incubated with [^{3}H]mevalonolactone, a precursor of isoprenoids, to verify that the 186Ser mutation of the chimera actually prevented prenylation. $G43_{10}61L$ did not incorporate label, although the v-H-Ras protein was successfully labeled under the same conditions (data not shown).

G43:Ras61L Is Palmitoylated at both Its N- and C-Terminii. $G43_{10}61L$ retains the two C-terminal cysteines at positions 181 and 184 which are the natural sites of palmitoylation in H-Ras. Thus, it was possible that palmitoylation of the cysteines from the GAP-43 leader and subsequent membrane association of the N-terminus might allow juxtaposition of the C-terminus to the membrane and permit the native C-terminal sites to become acylated. To explore this possibility, N- and C-terminal, [^{3}H]palmitate-labeled peptides derived from $G43_{10}61L$ and H-Ras61L proteins were compared. The peptides were generated by treatment of immunoprecipitated proteins with endoprotease Glu-C (*Staphylococcus aureus* V8 endoprotease), which cleaves exposed polypeptide bonds at the carboxy-terminal side of glutamic acid residues. As previously demonstrated for v-H-Ras (*26*), protease-treated H-Ras61L contained three peptides that were labeled by [^{3}H]palmitate, with apparent molecular masses of 21, 15, and 14 kDa (Figure 2B). The 21 kDa band was the full-length H-Ras61L, and decreased in amount in the lanes containing more protease. The 14 and 15 kDa bands corresponded to digestion products which contained the (palmitoylated) carboxy terminus of the H-Ras61L protein, and the amounts of these peptides increased with increasing amounts of protease. Previous experiments using [^{35}S]cysteine-labeled v-H-Ras protein (*5*) had shown that this procedure also yields a 7 kDa fragment that contains the (nonpalmitoylated) amino-terminal region of the H-Ras protein. No ^{3}H-labeled 7 kDa band was detected, because the amino terminus of H-Ras61L is not modified by palmitate. However, the absence of [^{3}H]-palmitate-derived label in this fragment indicated that [^{3}H]-palmitate had not been metabolized and reincorporated as amino acids. Furthermore, the labeling of the proteins had been done in the presence of cycloheximide, which allowed incorporation of radiolabel only into those proteins which had been synthesized previously. The protein-attached radiolabel thus remained as lipid.

In the case of $G43_{10}61L$, four distinct palmitoylated polypeptides appeared, with apparent molecular masses of 22, 21, 15, and 14 kDa (Figure 2B). The 22 kDa band corresponded to the full-length $G43_{10}61L$ protein, and was abundant in the lane containing the least amount of protease. In the lane containing intermediate amounts of protease, this band was replaced by a [^{3}H]palmitate-labeled 21 kDa band, corresponding to the apparent molecular mass of a H-Ras61L protein with an intact carboxy terminus but lacking the N-terminal GAP-43 leader. Because there are no Glu residues in the GAP-43 leader, the leader was presumably

removed by cleavage at one of the several Glu residues (positions 3, 31, 37) in the H-Ras amino terminus, generating a palmitoylated, N-terminal fragment too small to be retained on the gel. The 14 and 15 kDa [^{3}H]palmitate-labeled bands from G43$_{10}$61L had precisely the same mobility as their counterparts in the H-Ras61L sample, and also increased in intensity with increasing amounts of protease, suggesting that the same C-terminal cysteines were modified. The same pattern of fragments was also generated from the G43$_{11}$61L proteins (data not shown). Although [^{3}H]palmitate labeling of the N-terminal GAP-43 leader was not detectable by this technique, the presence of palmitate(s) at these cysteines was inferred by the retention of membrane binding when the C-terminal cysteines were replaced by serines (see Figure 3A). The presence of palmitate at the N-terminus was also implied by the loss of membrane binding and activity of another mutant, G43$_{10}$61L(*C3,4S*), which lacked the cysteines at positions 3 and 4 (see below). These data suggested that the chimeric G43:Ras61L protein was palmitoylated at cysteines in both N- and C-terminal domains.

Association of G43:RAS61L with Membranes. The ability of the (possibly four) palmitates present in the chimeric G43$_{10}$61L protein to support membrane binding of the cytosolic H-Ras(186Ser) protein was examined in more detail. The chimeric G43$_{10}$61L protein was found to be approximately 60% in the S-100 fraction and 40% in the P-100 in COS-1 cells (data not shown); a similar proportion of membrane-bound G43$_{10}$61L was also observed in transfected PC12 cells (Figure 3A). This percentage of membrane binding is similar to that seen with a GAP43−β-galactosidase fusion protein (*17*), which was found to be ~50% membrane-bound. As expected, the native lipidated form of H-Ras61L was entirely particulate. The GAP-43 leader sequence has three basic residues in addition to the sites for palmitoylation. These residues appeared to play only a minor role in the maintenance of membrane binding of G43$_{10}$61L, as washing the membranes of the P-100 fraction with 0.5 M NaCl to release protein bound through ionic interactions did not release any appreciable amount of the chimeric protein (data not shown). In addition, the G43$_{10}$61L(*C3,4S*) protein, which had these basic residues but which could not be palmitoylated at the amino terminus, was >90% soluble (Figure 3A).

If the population of protein molecules observed in the P-100 fraction partitioned there by virtue of the lipophilic character of the chimeric protein, then the protein should be released into solution upon solubilization of the membranes by the detergent Nonidet P-40 (NP-40). Figure 3B shows the result of resuspending the P-100 (100000*g* pellet) fraction in 1% NP-40 and separating the detergent-insoluble material from the detergent-soluble portion by centrifugation. The precursor of endogenous H-Ras was present in the soluble (S1) fraction, while mature endogenous H-Ras was found entirely in the NP-40-soluble fraction (S2) that was released from membranes. G43$_{10}$61L was found in all three fractions, including the NP-40 insoluble fraction. As had been found in COS-1 and PC12 cells, in NIH 3T3 cells G43$_{10}$61L was ~60% soluble. Approximately 30% of the chimeric protein partitioned with membranes and could be extracted by NP-40 (S2), while the remaining 10% partitioned with membranes but was not released by NP-40 (P). This behavior is reminiscent of full-length, native GAP-43, which displays a natural cytosolic pool of protein, and in which ~20% of the

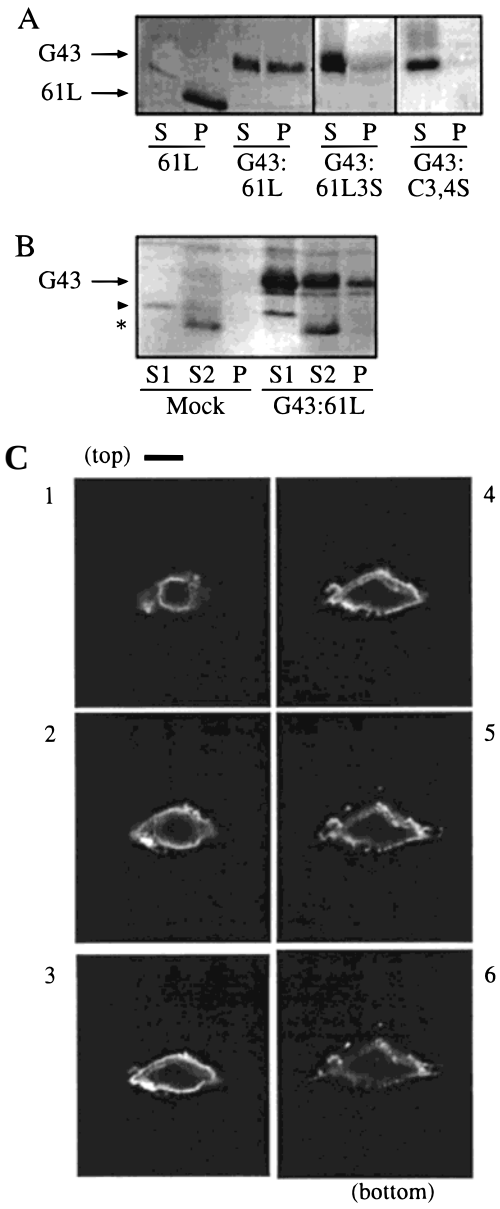

FIGURE 3: (A) G43$_{10}$61L associates with membranes. Subcellular membrane-containing (P) and soluble (S) fractions were prepared from PC12 cells transfected with 1 μg of pcDNA3 vectors encoding HRas61L [61L], G43$_{10}$61L, G43$_{10}$61L(*3Ser*) [G43:61L3S], or G43$_{10}$61L(*Cys3,4,Ser*) [G43:C3,4S]. Proteins were resolved by SDS−PAGE and H-Ras proteins detected by immunoblotting. (B) G43$_{10}$61L can be released from the P-100 membrane-containing fraction by NP-40. Subcellular fractions were prepared from NIH 3T3 cells that were untransfected or expressing G43$_{10}$61L. The cytosol (S1) was removed, the membrane-containing fraction was treated with NP-40, and the released NP-40 soluble proteins (S2) were separated from the NP-40 insoluble proteins (P). Proteins were separated by SDS−PAGE and detected by immunoblotting. In mock-transfected cells, the lipid-modified and trimmed endogenous H-Ras protein appears only in the detergent-solubilized, S2 fraction (asterisk) while the unmodified precursor of the endogenous H-Ras protein appears only in the soluble fraction (arrowhead); G43$_{10}$-61L (arrow) appears in all three fractions. (C) G43$_{10}$61L is targeted specifically to plasma membranes. Serial sections of a transfected COS-1 cells expressing G43$_{10}$61L protein, viewed by confocal immunofluorescent microscopy, show that nearly all of the fluorescence signal is present at the plasma membrane of the cell. Transfected COS-1 cells were plated on coverslips, fixed, permeablized, and incubated with monoclonal H-Ras antibody, followed by FITC-conjugated secondary antibody. The scale bar is 25 μm long.

Table 1: G43:Ras61L Proteins Are Weakly Transforming but Have Strong Differentiating Activity

DNA transfected	foci[a]/1000 colonies	relative transformation	% PC12 cells[b] without growth	relative differentiation
H-Ras61L	1250	1.00	54	1.0
$G43_{10}61L$	21	0.02	57	1.0
$G43_{10}61L$(*3Ser*)	nd[c]	nd	36	0.6
$G43_{106}1L$(*C3,4S*)	nd	nd	6	0.1
vector	3	0	<1	0

[a] Replicate 60 mm dishes of NIH 3T3 cells were transfected with 250 ng of $G43_{10}61L$ DNA or 25 ng of H-Ras61L DNA in the pZIP-SV(x)neo vector and were examined for either focus formation or growth of colonies in G418. Data are from one transfection, with $n = 6$ dishes each for $G43_{10}61L$ and $n = 3$ for pZIP vector only and H-Ras61L. Foci per dish were counted and divided by the number of colonies observed on a duplicate dish used for G418 selection. The H-Ras61L DNA gave 930 foci/μg of DNA. Similar results were observed in 5 independent transfections with amounts of $G43_{10}61L$ DNA ranging from 25 to 1000 ng. [b] PC12 cells were grown on laminin-coated dishes and transfected with 1 μg [3 μg for $G43_{10}61L$(*3Ser*)] of the indicated DNAs, all in the pcDNA3 vector. Four days later, >200 cells were counted and scored as differentiated if the cell displayed at least 1 outgrowth longer than 2 diameters of an untransfected cell (~100 μm). The experiment was replicated 4 times with similar results. [c] nd = not determined.

membrane-associated form is Triton-insoluble (*19*). Further studies will be necessary to determine if the Triton-insoluble population of acylated G43:Ras61L is simply denatured or is associated with specific, submembrane domains or structures (*26*).

G43:RAS61L Is Localized Specifically at the Plasma Membrane. Since there is no isoprenoid lipid attached to $G43_{10}61L$, the membrane association of the protein is presumably due to the presence of palmitate on the protein. However, the in vivo turnover and in vitro lability of the thioester bond by which the palmitate is attached make results from biochemical fractionation inherently uncertain. As a second way to assess membrane attachment, immunofluorescence was used to visualize the subcellular location of this protein in an intact cell. COS-1 cells which had not taken up DNA but were present on the same slide as transfected cells served as negative controls; the low amounts of endogenous H-Ras in untransfected COS-1 cells gave no detectable immunofluorescence signal. A series of confocal sections of a COS-1 cell (Figure 3C) or of PC12 cells (Figure 5) expressing $G43_{10}61L$ exhibited a clear, defined signal of H-Ras staining at the plasma membrane. No staining of internal membranes was observed. These data, taken with the biochemical data described above, strongly indicate that $G43_{10}61L$ is partially palmitoylated, but not isoprenylated, and that the portion of the chimeric protein which binds to membranes is located specifically at the plasma membrane.

G43:RAS61L Transforms NIH 3T3 Cells Poorly. The ability of the palmitoylated, nonisoprenylated G43:Ras61L protein to support cellular transformation was determined by transfection of NIH 3T3 cells and assessment of focus formation and growth in soft agar. Table 1 shows the relative transforming activities of the chimeric proteins. The ability of $G43_{10}61L$ to form foci in NIH 3T3 cells was detectable, but very low. Expression of the chimeric protein in cells derived from foci or G418-resistant colonies was confirmed by immunoblotting (data not shown). In comparison to the highly oncogenic H-Ras61L, the $G43_{10}$Ras61L protein caused

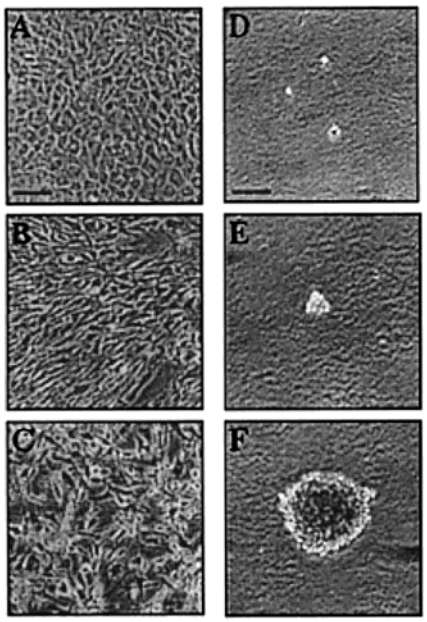

FIGURE 4: $G43_{10}61L$ causes changes in NIH 3T3 cell morphology and can support growth of NIH 3T3 cells in soft agar. (Left panels) G418-selected NIH 3T3 cells were transfected with pZip-neoSV-(x)1 vector DNAs encoding the indicated proteins. Individual colonies displaying representative cell morphologies were photographed. Scale bar is 100 μm. (A) Cells transfected with empty pZIPneo vector; (B) $G43_{10}61L$; (C) H-Ras61L. (Right panels) G418-selected NIH 3T3 cell lines expressing the indicated forms of H-Ras were seeded in 0.4% soft agar in DMEM and serum. At 2 weeks, colonies of average size for each cell line were photographed. Scale bar is 100 μm. (D) NIH 3T3 cells transfected with empty pZIPneo vector; (E) $G43_{10}61L$; (F) v-H-Ras.

only 2% as many foci. $G43_{10}61L$ protein also showed poor focus-forming activity (<5% of H-Ras61L) in Rat1 cells (data not shown).

NIH 3T3 cells expressing activated H-Ras proteins which contain both farnesyl and palmitoyl lipid modifications (e.g., H-Ras61L or v-H-Ras) exhibit a "transformed" morphology, characterized by an elongated, spindle-like cell shape and disorderly piling of cells in the culture dish; untransfected cells are flatter and rounder and form an orderly monolayer. The phenotype of G418-selected NIH 3T3 cells expressing $G43_{10}61L$ was intermediate between transformed and normal: cells were elongated, failed to spread on the culture dish, grew unchecked by cell−cell contact, and formed a partially disordered monolayer (Figure 4, left panels).

To resolve more rigorously if the modest morphologic changes and focus formation caused by $G43_{10}$Ras61L were evidence of genuine transformation, G418-selected cells were seeded in soft agar. Cell lines derived from $G43_{10}$Ras61L-expressing NIH 3T3 cells were able to form small colonies in soft agar while untransfected NIH 3T3 cells were not, and v-H-Ras-transformed cells formed large colonies (Figure 4, right panels). Together, these assays provide evidence that $G43_{10}$Ras61L can produce an authentic, but poorly transformed phenotype in NIH 3T3 mouse fibroblasts, but at a frequency much below that of an oncogenic H-Ras protein with both lipid modifications.

G43:Ras61L Induces Extension of Neurite-like Processes in PC12 Cells. PC12 cells expressing activated H-Ras proteins cease proliferation and undergo neuronal-like differentiation, similar to that observed when these cells are exposed to nerve growth factor (*27*). This biological effect,

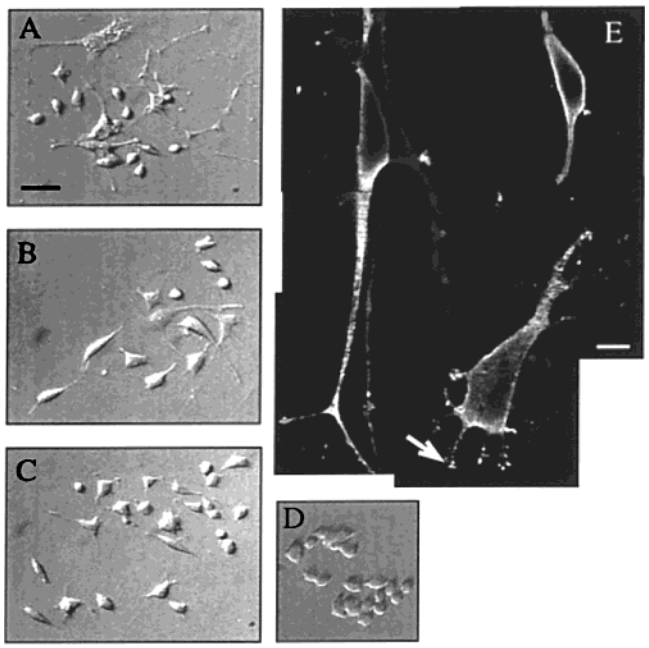

FIGURE 5: Expression of $G43_{10}61L$ causes differentiation of PC12 cells. PC12 cells were transfected with 1 μg each of pcDNA3 vectors encoding (A) H-Ras61L or (B and E) $G43_{10}61L$ or 3 μg of DNA for (C) $G43_{10}61L$(*3Ser*) and photographed 4 days later (5 days for panel C) using phase-contrast optics. Scale bar is 100 μm. In panel D, PC12 cells transfected with empty vector remain small and rounded and do not display membrane extensions. The immunofluorescence signal from the endogenous H-Ras in these cells was too weak to be reproduced photographically. For panel E, cells were prepared for immunofluorescence detection of H-Ras proteins as described under Materials and Methods, and then visualized by confocal immunofluorescence microscopy. Scale bar is 25 μm. A montage of images of a cell expressing $G43_{10}61L$ has been assembled to show the extent of cell elongation. The cell on the right displays bright staining of the ends of the numerous shorter extensions (arrow).

although quite different from the growth-promoting effects of activated H-Ras in NIH 3T3 cells, has also been shown to be dependent on membrane association of Ras (*16*, *28*). To determine how well the $G43_{10}61L$ protein might be able to trigger this distinct biological response, PC12 cells were transfected, and the outgrowth of neurite-like extensions was monitored. In contrast to the poor activity of $G43_{10}61L$ in focus formation, $G43_{10}61L$ elicited a strong morphological response in PC12 cells (Figure 5). $G43_{10}61L$-expressing PC12 cells developed axon-like outgrowths at the same rate and with the same frequency as cells expressing the fully lipidated H-Ras61L protein (Table 1). Thus, the chimera exhibited good differentiating activity in PC12 cells, indicating that there were no gross structural defects caused by the GAP43 leader that would explain its poor transformation performance. Biochemical fractionation of PC12 cells showed proportions of $G43_{10}61L$ in S-100 and P-100 fractions similar to those seen for COS-1 cell fractionations (data not shown). The outgrowths produced by the $G43_{10}61L$ protein did, however, display subtle differences from H-Ras61L-triggered extensions, having a smoother profile, with little ruffling of the cell body or along the extended outgrowth.

Using confocal immunofluorescence microscopy and an antibody to detect H-Ras proteins, all cells that were visibly differentiated (cells with extensions of >100 μm) contained chimeric protein. $G43_{10}61L$ chimeric protein was localized at the plasma membrane of the cell body as well as in the membranes of the axon-like outgrowths (Figure 5E). Staining was particularly prominent at the distal ends of outgrowths extending from the cells; veil-like sheaths of membrane spread around the cell body also stained very brightly. G43:Ras61L therefore was not trapped on internal membranes despite the absence of farnesyl (*29*), and was both targeted efficiently to the plasma membrane and highly active in PC12 cells.

C-Terminal Palmitoylation Contributes to the Biological Activity of G43:Ras. The unexpected reestablishment of palmitoylation of the C-terminal cysteines made it possible to examine what role lipids at the natural sites of modification were playing in the differentiating activity of the chimera. This was accomplished by constructing a new mutant $G43_{10}61L$(*3Ser*), in which the C-terminal cysteines, and their possibility for lipidation, were absent. When the $G43_{10}61L$(*3Ser*) protein was expressed in PC12 cells, there was clear differentiation, but this chimera caused fewer outgrowths (Figure 5C), which elongated at a much slower pace than the original $G43_{10}61L$ or H-Ras61L proteins (Table 1). Beyond 4 days post-transfection, the percentage of $G43_{10}61L$(*3Ser*)-transfected cells which displayed outgrowths did not increase; however, in cells which had outgrowths at 4 days, those extensions eventually did attain lengths equivalent to those caused by $G43_{10}61L$, although this process took 7−10 days, rather than the ∼4 days required normally after transfection of $G43_{10}61L$. Subcellular fractionation (Figure 3A) indicated that ∼20% of the $G43_{10}61L$(*3Ser*) protein was membrane-associated. In contrast, the $G43_{10}61L$(*C3,4S*) protein, in which only the two C-terminal sites were available for palmitoylation, showed very poor activity in the PC12 cells (Table 1) and was <10% membrane-associated (Figure 3A). It therefore appeared that acylation of the C-terminal cysteines and efficient membrane binding depended on establishment of N-terminal palmitoylation. Thus, the GAP-43 leader, if palmitoylated, could initiate and maintain a modest amount of membrane binding and allow the $G43_{10}61L$(*3Ser*) protein to cause PC12 cell differentiation, but $G43_{10}61L$(*3Ser*)-supported differentiation was less efficient than $G43_{10}61L$. These biological results supported the biochemical evidence of palmitates at the C-terminus of the original $G43_{10}61L$. The C-terminal lipids were therefore implicated as contributing a tangible portion of the binding of $G43_{10}61L$ with membranes and its ability to produce differentiation of PC12 cells.

DISCUSSION

Dynamic Acylation Can Sustain Partial H-Ras Membrane Binding. These results demonstrate that, despite their biological turnover, palmitates are capable of sustaining H-Ras−membrane interaction without assistance from a permanently attached lipid or polybasic domain. Thus, the G43:Ras61L proteins show that dynamic acylation is competent to perform its postulated role in membrane binding. However, the significant limitations in membrane binding of the nonfarnesylated G43:Ras61L proteins (none were better than 40% membrane-bound) also make it clear that in the native state H-Ras needs both farnesyl and C-terminal palmitates to achieve a high level of membrane association. This suggests that in native H-Ras C-terminal palmitates may not assume constant responsibility for membrane interaction, and that support from farnesyl may continue to be needed. G43:

Ras61L will be useful for examining if palmitate removal/attachment sets a threshold or limits the H-Ras accumulation on membranes.

Requirements for C-Terminal Palmitate Attachment and Membrane Targetting. It had not been expected that G43:Ras61L would be palmitoylated at the natural C-terminal sites. Rigorous analysis of N-myristoylated H-Ras forms had not detected palmitate if short labeling periods or cycloheximide was used to eliminate incorporation of radiolabel as myristate (*11, 14*). Authentic C-terminal palmitoylation had been accomplished using basic residues attached C-terminal to the C*aa*X motif of yeast Ras2 or H-Ras (*16, 30*), but G43:Ras61L is the first design to accomplish acylation of these sites in a C-terminus with only a single amino acid change (186Ser). However, even with four sites available for palmitate attachment, 60% of $G43_{10}61L$ remained cytosolic, indicating that steady-state palmitoylation of $G43_{10}61L$ was less complete than for H-Ras that could be farnesylated. Farnesyl thus appears to be particularly effective for positioning H-Ras in locations or configurations to optimize palmitate attachment and retention. Recent studies report that farnesyl may target Ras proteins to the cytoplasmic face of the endomembrane system, at which sites the proteins are further processed, perhaps palmitoylated, and then trafficked to the plasma membrane (*29*). As $G43_{10}61L$ appears to be efficiently localized at the plasma membrane, it will be useful for determining the roles of palmitate and farnesyl in this unexplored targeting pathway.

Palmitoylation by Itself Fails To Support Full H-Ras Function. Despite the presence of palmitates at its C-terminus and significant amounts of protein in the plasma membrane, the transformation efficiency of G43:Ras61L was seriously decreased. The potency of G43:Ras61L in PC12 cell differentiation demonstrated that its low transforming activity did not result from an intrinsic defect in G43:Ras61L structure caused by the GAP-43 sequences. This strength of G43:Ras61L differentiating activity also suggests that the substantial amounts of cytoplasmic chimera are not likely to impede signaling by potentially binding Ras effector proteins in the cytosol (*31*−*33*). The current results therefore provide the significant finding that transformation is particularly susceptible to an unexplored limitation that occurs during interaction of palmitoylated G43:Ras61L with membranes. It will be important to determine whether this sensitivity results from the absence of farnesyl or from a new, palmitate-dependent balance in general membrane binding or altered membrane subdomain targeting of the G43:Ras61L protein. The unusual inequality in transforming vs differentiating activity of G43:Ras61L may also help identify a subset of Ras effectors responsible for this desirable transformation-specific defect.

Roles of Lipids in Effector Interactions. This report and several others have shown that forms of H-Ras that have no C-terminal lipids can be biologically active (*11, 14, 34*); thus, neither palmitate nor farnesyl can be strictly necessary for the initial protein−protein interactions between H-Ras and its effectors. However, recent studies suggest that an interaction between the cysteine-rich (zinc finger) domain of Raf and the farnesyl-modified form of H-Ras is necessary for Raf activation (*35, 36*). Because nonpalmitoylated H-Ras with a farnesyl is 90% cytoplasmic, one function of palmitoylation appears to be to ensure membrane binding so that farnesylated H-Ras does not accumulate in the cytoplasm and potentially sequester crucial partner proteins. It remains to be seen if farnesyl, palmitate, or amino acids in the H-Ras C-terminal domain have any further roles in maximizing or stabilizing effector interactions at the membrane. Either farnesyl or palmitate could also regulate interactions of H-Ras with subdomains of membranes that have unique lipid compositions [e.g., caveolae (*26, 37*−*40*)]. The ability to utilize intact cells to produce G43:Ras61L proteins, with a palmitoylated C-terminus whose structure and interactions are as close as possible to native, while specifically lacking isoprenoid, should be useful for testing both models.

The Unique Role of Palmitate. The dynamic nature of H-Ras palmitoylation is its clearest distinction from farnesylation and likely forms a crucial part of palmitate's character. There is a growing appreciation that reversible palmitoylation may regulate key signal transduction pathways (*41*−*43*). The endothelial form of nitric oxide synthase responds to bradykinin with an increase in palmitate turnover (*44, 45*). β-Adrenergic receptor palmitoylation is sensitive to isoproterenol treatment and appears to influence receptor phosphorylation and desensitization (*46, 47*). Palmitoylation of α subunits of several heterotrimeric G proteins is both dynamic and responsive to hormonal signals (*48*−*54*). Palmitoylation of the α subunit of the G_z protein inhibits interaction with its GAP protein and may prolong or potentiate signaling by this protein (*55*). In these examples, palmitate is more than a passive passenger on the acyl-protein; it is potentially an active participant in the signaling cycle. The G43:Ras61L proteins provide an opportunity to learn if palmitate, in addition to its task of membrane binding, plays this role in signaling in H-Ras.

Although a great deal of effort has been given to development of inhibitors of isoprenoid modification as a potential means to control the unwanted oncogenicity of Ras proteins, our findings focus attention on control of palmitoylation as an opportunity for significant regulation of these proteins. The development of G43:Ras61L proteins should now allow studies to more clearly define the unique roles of palmitate and isoprenoid in Ras biological function.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Dr. Phil Haydon for confocal microscopy; and Molly Whedby, Luke McConeghy, and Kelly Welch for assistance with COS-1 and PC12 cell transfections and preparation of subcellular fractions from various mutant G43:Ras61L proteins. We also thank Deborah Brown, J. H. Pate Skene, and John Stickney for helpful comments on the manuscript.

REFERENCES

1. Gutierrez, L., Magee, A. I., Marshall, C. J., and Hancock, J. F. (1989) *EMBO J. 8*, 1093−1098.
2. Casey, P. J., Solski, P. A., Der, C. J., and Buss, J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8323−8327.
3. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) *Cell 57*, 1167−1177.
4. Zhang, F. L., and Casey, P. J. (1996) *Annu. Rev. Biochem. 65*, 241−269.
5. Buss, J. E., and Sefton, B. M. (1986) *Mol. Cell. Biol. 6*, 116−122.
6. Lu, J.-Y., and Hofmann, S. L. (1995) *J. Biol. Chem. 270*, 7251−7256.

7. Magee, A. I., Gutierrez, L., McKay, I. A., Marshall, C. J., and Hall, A. (1987) *EMBO J. 6*, 3353−3357.
8. Buss, J. E., and Marsters, J. C., Jr. (1995) *Chem. Biol. 2*, 787−791.
9. Cox, A. D., and Der, C. J. (1992) *Curr. Opin. Cell Biol. 4*, 1008−1016.
10. Gibbs, J. B., Oliff, A., and Kohl, N. E. (1994) *Cell 77*, 175−178.
11. Cadwallader, K. A., Paterson, H., MacDonald, S. G., and Hancock, J. F. (1994) *Mol. Cell. Biol. 14*, 4722−4730.
12. Dudler, T., and Gelb, M. H. (1996) *J. Biol. Chem. 271*, 11541−11547.
13. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) *Cell 63*, 133−139.
14. Willumsen, B. M., Cox, A. D., Solski, P. A., Der, C. J., and Buss, J. E. (1996) *Oncogene 13*, 1901−1909.
15. Shahinian, S., and Silvius, J. R. (1995) *Biochemistry 34*, 3813−3822.
16. Booden, M. A., Baker, T. L., Solski, P. A., Der, C. J., Punke, S. G., and Buss, J. E. (1999) *J. Biol. Chem. 274*, 1423−1431.
17. Zuber, M. X., Strittmatter, S. M., and Fishman, M. C. (1989) *Nature 341*, 345−348.
18. Liu, Y., Fisher, D. A., and Storm, D. R. (1993) *Biochemistry 32*, 10714−10719.
19. Skene, J. H. P., and Virag, I. (1989) *J. Cell Biol. 108*, 613−624.
20. Wolven, A., Okamura, H., Rosenblatt, T., and Resh, M. D. (1997) *Mol. Biol. Cell. 8*, 1159−1173.
21. Solski, P. A., Quilliam, L. A., Coats, S. G., Der, C. J., and Buss, J. E. (1995) *Methods Enzymol. 250*, 435−454.
22. Willumsen, B. M. (1995) *Methods Enzymol. 250*, 269−283.
23. Buss, J. E., Solski, P. A., Schaeffer, J. P., MacDonald, M. A., and Der, C. J. (1989) *Science 243*, 1600−1603.
24. Mumby, S., and Buss, J. E. (1990) *Methods: Companion Methods Enzymol. 1*, 216−220.
25. Hart, K. C., and Donoghue, D. J. (1997) *Oncogene 14*, 945−953.
26. Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G., and Brown, D. A. (1999) *J. Biol. Chem. 274*, 3910−3917.
27. Sassone-Corsi, P., Der, C. J., and Verma, I. (1989) *Mol. Cell. Biol. 9*, 3174−3183.
28. Qiu, S. D., Pitts, A. F., Winters, T. R., and Green, S. H. (1991) *J. Cell Biol. 115*, 795−808.
29. Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E., and Philips, M. R. (1999) *Cell 98*, 69−80.
30. Mitchell, D. A., Farh, L., Marshall, T. K., and Deschenes, R. J. (1994) *J. Biol. Chem. 269*, 21540−21546.
31. Miyake, M., Mizutani, S., Kiode, H., and Kaziro, Y. (1996) *FEBS Lett. 378*, 15−18.
32. Lerner, E. C., Qian, Y., Blaskovich, M. A., Fossum, R. D., Vogt, A., Sun, J., Cox, A. D., Der, C. J., Hamilton, A. D., and Sebti, S. M. (1995) *J. Biol. Chem. 270*, 26802−26806.
33. Gibbs, J. B., Schaber, M. D., Schofield, T. L., Scolnick, E. M., and Sigal, I. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6630−6634.
34. Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G., and Sefton, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7493−7497.
35. Roy, S., Lane, A., Yan, J., McPherson, R., and Hancock, J. F. (1997) *J. Biol. Chem. 272*, 20139−20145.
36. Luo, Z., Diaz, B., Marshall, M. S., and Avruch, J. (1997) *Mol. Cell. Biol. 17*, 46−53.
37. Mineo, C., Anderson, R. G. W., and White, M. A. (1997) *J. Biol. Chem. 272*, 10345−10348.
38. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem. 271*, 9690−9697.
39. Galbiati, F., Volonte, D., Meani, D., Milligan, G., Lublin, D. M., Lisanti, M. P., and Parenti, M. (1999) *J. Biol. Chem. 274*, 5843−5850.
40. Roy, S., Luetterforst, R., Harding, A., Apollini, A., Etheridge, M., Stang, E., Rolls, B., Hancock, J. F., and Parton, R. G. (1999) *Nat. Cell Biol. 1*, 97−105.
41. Milligan, G., Parenti, M., and Magee, A. I. (1995) *Trends Biochem. Sci. 20*, 181−186.
42. Mumby, S. M. (1997) *Curr. Opin. Cell Biol. 9*, 148−154.
43. Dunphy, J. T., and Linder, M. E. (1998) *Biochim. Biophys. Acta 1436*, 245−261.
44. Robinson, L. J., Busconi, L., and Michel, T. (1995) *J. Biol. Chem. 270*, 995−998.
45. Liu, J., Garcia-Cardena, G., and Sessa, W. C. (1995) *Biochemistry 34*, 12333−12340.
46. Moffett, S., Adams, L., Bonin, H., Loisel, T. P., Bouvier, M., and Mouillac, B. (1996) *J. Biol. Chem. 271*, 21490−21497.
47. Loisel, T. P., Adam, L., Hebert, T. E., and Bouvier, M. (1996) *Biochemistry 35*, 15923−15932.
48. Huang, C., Duncan, J. A., Gilman, A. G., and Mumby, S. M. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 412−417.
49. Degetyarev, M. Y., Spiegel, A. M., and Jones, T. L. Z. (1993) *J. Biol. Chem. 268*, 23769−23772.
50. Iiri, T., Backlund, P. S., Jr., Jones, T. L. Z., Wedegaertner, P. B., and Bourne, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14592−14597.
51. Mumby, S. M., Kleuss, C., and Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2800−2804.
52. Wedegaertner, P. B., and Bourne, H. R. (1994) *Cell 77*, 1063−1070.
53. Wedegaertner, P. B., Wilson, P. R., and Bourne, H. R. (1995) *J. Biol. Chem. 270*, 503−506.
54. Bhamre, S., Wang, H. Y., and Friedman, E. (1999) *J. Pharmacol. Exp. Ther. 286*, 1482−1489.
55. Tu, Y., Wang, J., and Ross, E. M. (1997) *Science 278*, 1132−1135.

BI9909290