Ha-Ras undergoes post-translational modifications (including attachment of farnesyl and palmitate) that culminate in localization of the protein to the plasma membrane. Because palmitate is not attached without prior farnesyl addition, the distinct contributions of the two lipid modifications to membrane attachment or biological activity have been difficult to examine. To test if palmitate is able to support these crucial functions on its own, novel C-terminal mutants of Ha-Ras were constructed, retaining the natural sites for palmitoylation, but replacing the C-terminal residue of the CAAX signal for prenylation with six lysines. Both the Ext61L and ExtWT proteins were modified in a dynamic fashion by palmitate, without being farnesylated; bound to membranes modestly (40% as well as native Ha-Ras); and retained appropriate GTP binding properties. Ext61L caused potent transformation of NIH 3T3 cells and, unexpectedly, an exaggerated differentiation of PC12 cells. Ext61L with the six lysines but lacking palmitates was inactive. Thus, farnesyl is not needed as a signal for palmitate attachment or removal, and a combination of transient palmitate modification and basic residues can support Ha-Ras membrane binding and two quite different biological functions. The roles of palmitate can therefore be independent of and distinct from those of farnesyl. Reciprocally, if membrane association can be sustained largely through palmitates, farnesyl is freed to interact with other proteins.

In its GTP-bound conformation, Ha-Ras activates several signal transduction cascades that control gene expression and actin cytoskeleton organization. The most well studied of these pathways, the Raf/mitogen-activated protein kinase cascade, involves a series of cytoplasmic serine/threonine kinases; another utilizes several members of the Rho family of GTP-binding proteins that regulate cell morphology. In NIH 3T3 mouse fibroblasts, activated forms of Ha-Ras induce mitogenesis and transformation; in the rat pheochromocytoma cell line PC12, Ha-Ras activation triggers neuronal differentiation. More important, membrane localization of Ha-Ras is critical for either of these distinct biological activities. One important consequence of membrane binding is to enable Ha-Ras to act as a GTP-dependent, membrane-localized docking site for effector proteins, such as Raf kinase.

The correct targeting of Ha-Ras to the inner surface of the plasma membrane requires a series of post-translational modifications at the protein’s C terminus. These reactions include attachment of the isoprenoid farnesyl to a cysteine residue (Cys-186) located four residues from the C terminus, followed by removal of the C-terminal tripeptide and methylation of the newly exposed carboxyl group of the farnesylated cysteine (1). The final step is palmitoylation of two nearby C-terminal cysteines (Cys-181 and Cys-184) (2, 3).

The duties of these lipids has been inferred principally from mutant Ras proteins that lack the palmitates, but retain the isoprenoid, or that lack all three lipids entirely. The decreased membrane binding observed with these modification mutants has led to the model that membrane binding is a primary role for the lipids attached to Ha-Ras. However, the function(s) of farnesyl and palmitate, individually, have not been completely elucidated.

The consequences of farnesyl attachment are particularly important because prenylation is the first step in processing, putting farnesylattachment in temporal control of subsequent modifications. Without an isoprenoid attached to Cys-186, the Ha-Ras protein remains soluble, and the adjacent Cys-181 and Cys-184 do not become palmitoylated. An isoprenoid has been shown to be a prerequisite modification for recognition by both the membrane-bound protease and methylase enzymes, which further modify the C terminus (4, 5). It has been suggested that a putative palmitoyl acyltransferase may also require the presence of a farnesyl group (6), but the authenticity of this enzymatic activity has not been confirmed. Farnesyl thus functions in maturation of Ras proteins by acting as part of a signal sequence for and allowing interaction of Ras with at least two membrane-bound processing enzymes.

The most well recognized role for farnesyl is its participation in Ras membrane binding. Prenylation is required for initiating the transition of the cytosolic precursor to the membrane-bound form. Whether farnesyl also plays a role in sustaining or directing submembrane localization has not been clarified. Farnesyl by itself does not confer high affinity membrane binding to Ras peptides in vitro (7–9), nor does it suffice for targeting of the protein to plasma membranes in intact cells (10–12). The farnesyl group appears to depend heavily on a second mechanism to assist its efforts in membrane binding. Two types of “secondary” membrane-binding signals have been identified: a hydrophobic type involving palmitoylation of nearby cysteine residues, as is found in Ha-Ras, N-Ras, and Ki-Ras4A; and an ionic type, based on the series of basic residues just N-terminal of the CAAX sequence, as found in Ki-Ras4B (2, 13).

A number of studies have now suggested that farnesyl may have an additional role besides initiating membrane attach-
ment. The farnesyl group is proposed to bind to specific membrane proteins (14), a process that might enhance Ras/membrane interaction or that might be needed for activation of Ras effector proteins. Good evidence has been presented that a structural or conformational epitope found in the prenylated form of Ha-Ras is important (in addition to the role of Ras in Raf membrane targeting) for activation of Raf-1 through the kinase’s cysteine-rich zinc finger domain (15–17). In vitro analysis has suggested that farnesylation of Ha-Ras may also be needed for human SOS1 to promote guanine nucleotide exchange (18). Earlier studies using an activated yeast Ras2 protein revealed that mutation of the farnesylation site decreased the interaction between Ras2 and adenylcyclase (19, 20). Thus, these studies imply that the prenyl structure of Ras may be involved in and perhaps required for specific associations of Ha-Ras and its regulatory or target proteins. How potential roles in both lipid bilayer binding and protein interactions can be performed simultaneously or sequentially remains an important, unanswered question.

The duties for which the palmitates of Ha-Ras are used are much less clear. Palmitate certainly has sufficient hydrophobicity to support membrane binding of an Ha-Ras protein, but the requirement for prior farnesylation has prevented study of the role(s) of the Ha-Ras palmitates independent of isoprenoid. It is possible that palmitoylation of Cys-181 and Cys-184 of Ha-Ras is only a nonessential (although required) secondary membrane attachment signal that simply enables more substantial or sustained levels of membrane binding of a farnesylated Ha-Ras protein. More direct, biochemical studies of palmitoylation have been thwarted because the enzymes that might attach palmitates to Ha-Ras, or similar proteins, have proved to be extremely difficult to isolate (6, 21, 22). In vivo requirements for palmitate attachment have been examined in a large series of C-terminal mutant Ha-Ras proteins, all of which became palmitoylated (11). These studies identified amino acids that appeared to be novel signals for intracellular trafficking, but no consensus sequence for palmitate attachment was found. However, these mutant proteins still retained an intact CAAX motif and were farnesylated, so it remained possible that farnesyl might be part of an otherwise enigmatic signal for palmitoylation. Because several of these farnesylated and palmitoylated mutant Ha-Ras proteins were mis-localized to internal membranes within the cell, one clear result was that acquisition of both C-terminal lipids is not sufficient to assure correct plasma membrane targeting.

However, based on observations that C181S/C184S mutants of Ha-Ras (which retain farnesyl but lack palmitate) localize poorly to the plasma membrane, several studies have suggested that it is palmitate rather than farnesyl that is largely responsible for Ha-Ras membrane binding. In the Xenopus oocyte maturation assay and also in transformation of NIH 3T3 cells, Ha-Ras proteins modified only by farnesyl have poor biological activity, indicating that acquisition of palmitates is important functionally (9–11, 23, 24). Using a yeast plasmid-loss assay, Mitchell et al. (25) studied a series of yeast Ras2 proteins that showed that the combined effect of C-terminal basic amino acids and palmitoylation of cysteine residues was sufficient to support Ras-dependent growth, independent of prenylation. More important, the biological function of these yeast Ras2 proteins correlated with their ability to be palmitoylated. These results show that palmitate is an important contributor to Ha-Ras membrane binding and function and imply that, despite its chronological precedence, farnesyl depends upon palmitate to support these activities. However, it remains unclear whether palmitate mutually requires the farnesyl or plays an independent but complementary role in specific membrane association and biological activity of Ha-Ras. To enable study of requirements for palmitate addition and to determine if palmitates could direct specific plasma membrane targeting, sustain Ha-Ras membrane binding, and support biological function, a novel mammalian Ha-Ras protein was constructed, designed to have C-terminal palmitates, but no isoprenoid. The results indicate that, although farnesyl needs the assistance of palmitate for membrane binding and full biological function, palmitates can support substantial farnesyl-independent activity. The results begin to define distinct roles for Ha-Ras farnesyl and palmitate and suggest that palmitate is more than just an energetic form of membrane tether that serves the needs of the farnesyl and may have unique and dynamic biological roles of its own.

MATERIALS AND METHODS

Mutants and Plasmids—Ext61L, ExtWT, Ext61L(C181S/C184S/C186S), ExtWT(C181S/C184S/C186S), and Ha-Ras61L were expressed from pcDNA3 (Invitrogen, Carlsbad, CA). To construct Ext61L, an S190K substitution followed by the addition of five lysine residues was introduced into wild-type Ha-Ras with a Q61L activating mutation by oligonucleotide-directed polymerase chain reaction mutagenesis. The construction used an oligonucleotide 5′-GGGGGGATCCATCATGATCAGAATAACAAGCTT−3′, which was fully complementary to the 5′-sequences of human Ha-Ras, and the mutagenic oligonucleotide 5′-GGGGGGATCCCATACTTCCTCTCTCTCTCTTTTTTAACACACTCTTA−3′, which reproduced the nucleotides complementary to the 3′-sequences of Ha-Ras and introduced the six lysine codons (underlined), a termination codon, and a BamHI restriction enzyme recognition site. The resulting pcDNA3-Ext61L cDNA was inserted as a BamHI fragment into the BamHI site of pcDNA3 and encoded the complete Ha-Ras protein with a C-terminal polylysine tail (Fig. 1A). pcDNA3-ExtWT is identical to pcDNA3-Ext61L, except that it encodes the non-activated cellular form of Ha-Ras with the normal glutamine residue at position 61. The resulting ExtWT(C181S/C184S/C186S) was constructed in a similar fashion using the mutagenic oligonucleotide 5′-GAATTCCTAGATCAGACCTGATCGACACAATCCTGA−3′ to introduce the six lysine codons (underlined) and to change codons 181, 184, and 186 from Cys to Ser (double underlined). The cDNA was inserted as a BamHI-NotI fragment into pcDNA3.

Cell Culture, Transfection, and Transformation Assays—PC12 cells were maintained at 5% CO2 in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum (both from Hyclone Laboratories, Logan, UT). Twenty-four hours before DNA transfection, PC12 cells were plated onto laminin (10 μg/ml) and Life Technologies, Inc.)-coated 60-mm tissue culture dishes at a density of 105 cells/dish. Transfection was performed using the calcium phosphate precipitation technique (11, 26). Transfected cells were also selected in growth medium containing G418 (Genetecin, Life Technologies, Inc.) at 500 μg/ml to establish cell lines that stably expressed the mutant proteins.

Preparation of Subcellular Fractions—Subcellular fractions were prepared by lysis of cells in hypotonic buffer (0.1 m Tris, pH 7.4, 0.5 mM MgCl2, 1 mM Pefabloc, 1 μM leupeptin, 2 μM peptatin, and 0.1% aprotinin (Calbiochem)) and addition of NaCl to adjust the ionic strength to 0.15 M, followed by ultracentrifugation for 30 min at 100,000 × g as described previously (26). Hydrophobic (e.g. lipid-modified) proteins were isolated by separation into detergent (hydrophobic) and aqueous (hydrophilic) fractions by Triton X-114 lysis and phase separation as described (2). For testing release of proteins from the P100 membrane fraction, the supernatant (S100 = S1) was set aside, and the pellet was resuspended in 1 ml of hypotonic buffer supplemented with 0.5 mM NaCl and incubated on ice for 30 min. The suspension was centrifuged at 100,000 × g; the supernatant (S2) was collected; and the pellet was incubated with the high salt buffer a second time and centrifuged, yielding a third supernatant (S3). The pellet from the second salt wash (P3) was resuspended in 1 ml of radioimmunoprecipitation assay buffer and centrifuged, and the radioimmunoprecipitation assay extract (S4) removed. The proteins in the separate S1–S4 cells.
supernatants were precipitated with 10 ml of acetone for 1 h at 4 °C, collected by centrifugation at 3000 rpm for 30 min, and dissolved in 100 µl of electrophoresis sample buffer. Equal volumes of each fraction were separated by SDS-PAGE.

Radioisotopic labeling—Cells were incubated with 200 µCi/plate Trans 3H-label (ICN, Costa Mesa, CA) in Dulbecco's modified Eagle's medium lacking cysteine/methionine for 18 h. To examine isoprenoid attachment, cells were pretreated with 30 µM compactin for 30 min and then labeled for 18 h with 100 µCi/ml [3H]mevalonolactone (American Radiolabeled Chemicals, St. Louis, MO) in the continued presence of compactin (27). Palmitate incorporation and subsequent depalmitoylation were measured for 1 h by labeling cells for 4 h with 1 mCi/ml [3H]palmitate (NEN Life Science Products) in medium containing nonessential amino acids, 25 µg/ml cycloheximide, and 10% calf serum and then incubating for varying times in nonradioactive medium containing 200 µM palmitic acid. After radioisotopic labeling, samples were prepared for subcellular fractionation as described above, or cells were lysed directly in high SDS/ribozyme precipitation assay buffer (50 mM Tris, pH 7.0, 0.5% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, and 50 mM aprotinin (Calbiochem) for immunoprecipitation. Extracts of cell fractions were incubated on ice for 1 h with Ha-Ras-specific mouse monoclonal antibody 146-3E4 (Quality Biotech Inc., Camden, NJ).

Immunocomplexes were recovered with Staphylococcus aureus cells (Pansorbin, Calbiochem) coated with goat anti-mouse heavy and light chain IgG; washed; resuspended in special sample buffer (2% SDS, 10 mM Na3VO4, pH 7.0, 10% glycerol, 0.05% dithiothreitol, and 0.02% bromphenol blue); resolved by SDS-PAGE; transferred to polyvinylidene difluoride membrane; and, as needed, sprayed with ENHANCE (NEN Life Science Products) for fluorography or developed for immunoblot analysis. For immunoblotting, after separation by SDS-PAGE, proteins were transferred electrochemically to polyvinylidene difluoride membranes, and nonspecific protein binding was blocked by incubating the membrane in 1.25% nonfat dry milk in Tris-buffered saline. Membranes were probed with Ha-Ras-specific monoclonal antibody 146-3E4. Biotinylated secondary antibodies (anti-mouse; Vector Laboratories, Inc., Burlingame, CA) were used with development by alkaline phosphatase (Vector Laboratories, Inc.) using the manufacturer’s protocol.

Immunofluorescence—PC12 cells were plated at low density on serum-coated coverslips in six-well dishes and transfected with Ext61L DNA as described above. Following fixation with freshly prepared 2% paraformaldehyde, cells were treated with a blocking solution containing 0.4% bovine serum albumin and 3% horse serum to block nonspecific antibody interactions and 0.05% Triton X-100 and 0.05% Tween 20 to permeabilize the cells (28). Cells were then treated with a 1:20 dilution of Ha-Ras-specific rat monoclonal antibody Y13-172, followed by a 1:50 dilution of fluorescein isothiocyanate-conjugated rabbit anti-rat secondary antibody (Cappel/Organon Teknika, Durham, NC) both diluted in blocking solution; mounted in diazabicyclo[2.2.2]octane/glycerol solution to prevent fading; and viewed by confocal microscopy.

GTP/GDP Determination—Confluent cultures of PC12 cells were grown overnight in 1% dialyzed calf serum. Cells were then radioisotopically labeled with 0.5–1 mCi/ml 32P1 (NEN Life Science Products) for 4 h in growth medium containing 1% calf serum. Cells were rinsed with phosphate-buffered saline and lysed in 0.6 ml of cold GTP assay buffer containing 50 mM Tris-HCl, pH 7.4, 20 mM MgCl2, 50 mM NaCl, 0.5% (v/v) Nonidet P-40, 20 µg/ml aprotinin, 1 mM EGTA, and 1 mM Na3VO4. Insoluble material was removed by centrifugation at 750 × g for 3 min. Supernatants were cleared with 50% (v/v) bovine serum albumin-coated charcoal in lysis buffer, and then Ha-Ras proteins were captured by immunoprecipitation using GTP assay buffer. Ha-Ras and its bound nucleotides were eluted by heating to 60 °C for 5 min in a minimal volume of buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2% (w/v) SDS, 2 mM GTP, and 2 mM GTP. The eluants were cleared by centrifugation at 10,000 × g for 5 min, and then samples were spotted on polyethyleneimine cellulose plates (J. T. Baker Inc.) and separated with 0.75 M KH2PO4, pH 3.4. Plates were dried and exposed to prefixed film using screen enhancers. The film images of GDP and GTP were scanned and quantified using the program ImageQuant (Molecular Dynamics, Inc.). Results were expressed as percentage of an Ha-Ras protein that contained bound GTP was calculated using the following formula: % with GTP = GTP/(GTP + 1.5 GDP).

RESULTS

Ext61L cDNA Produces A Stable Protein That Is Not Prenylated—Multiple studies that had examined a great many mutations within and including the N-terminal of the CAAX box of non-prenylated Ha-Ras or Ki-Ras4B proteins had all failed to detect membrane binding (a biochemical surrogate for potential palmitoylation). The only successful examples of palmitoylated forms that lacked isoprenoid were the yeast Ras2 mutants in which the CAAX box was extended with a series of basic amino acid residues (25). This work had suggested that lengthening and introducing positively charged residues enabled a yeast Ras2 protein to contact membranes and to become palmitoylated. The mammalian ExtRas proteins were therefore designed with a lysine in the X position of the CAAX box, which was anticipated would produce a nonfunctional prenylation motif, and five additional lysine residues to provide an ionic platform for interaction with acidic membrane phospholipids.

The initial experiments determined the stability and size of the Ext61L protein and whether the carboxy-terminal CV-LKKK sequence (Fig. 1A) prevented the Ext61L protein from being prenylated. This was important because it was possible the Ext61L protein would be vulnerable to proteolytic removal of these basic residues, which would remove the structure that was meant to initiate and assist membrane binding. Also, removal of five lysines would potentially recreate a CAAX box motif (CVLKK) that might permit prenylation. This was not considered likely as the presence of a lysine at the X position had previously been found to prevent prenylation of a Ki-Ras4B protein (29). If such trimming and prenylation were to occur, it could be detected because it would produce a smaller protein, similar in size to mature native Ha-Ras protein. An additional way to detect, indirectly, if prenylation was occurring was to treat cells with compactin. Compactin inhibits isoprenoid synthesis and hence decreases Ras prenylation, preventing the

The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
change in mobility and leading to the accumulation of the unmodified precursor form of Ha-Ras in the cytosol (1).

To test these possibilities, COS cells expressing Ext61L were labeled metabolically with [35S]methionine in the presence or absence of compactin. Cytosolic and membrane-containing fractions were prepared, and proteins were separated by SDS-PAGE and detected by fluorography. The Ext61L protein was easily detected and had the appropriate, slightly slower mobility than the endogenous Ha-Ras protein of COS cells, indicating that the C-terminal residues of Ext61L were retained (Fig. 1B). More important, a significant portion (40%) of the Ext61L protein was detected in the membrane-containing fraction and had the same apparent size as the cytosolic protein. This similarity in size of proteins in the S100 and P100 fractions indicated indirectly that the extension protein was not farnesylated. In addition, compactin did not decrease the amount of Ext61L protein that was present in the P100 fraction. Without compactin treatment, >90% of the small amount of endogenous Ha-Ras protein in untransfected or transfected COS cells (Fig. 1B) was found in the membrane fraction. In the compactin-treated cells, the amount of membrane-bound endogenous Ha-Ras decreased; the increased amount of the precursor form in the cytosol was obscured by the large amount of soluble Ext61L protein.

To evaluate more directly whether the Ext61L protein remained unmodified by isoprenoid, COS cells expressing this protein or Ha-Ras61L were labeled with [3H]mevalonic acid, and the Ras proteins were isolated by immunoprecipitation. No incorporation of radioactivity could be detected in the Ext61L lane (Fig. 1C, lane 2). However, labeling of cells expressing Ha-Ras61L confirmed that an Ha-Ras protein with an intact CAAX motif could be prenylated (Fig. 1C, lane 1). In addition, immunoblot detection of the same membrane confirmed that the Ext61L protein was expressed (data not shown). These results indicated that the Ext61L protein was not prenylated.

**Ext61L Binds Membranes Rapidly and Is Targeted to the Plasma Membrane**—Although a significant amount of Ext61L protein was found in the membrane-containing fraction in the cells, the extent of binding of the Ext61L protein was far less than the >95% binding attained by endogenous Ha-Ras with the natural version of the C terminus. Previously constructed C-terminal mutants of v-Ha-Ras had also shown decreased or delayed membrane binding compared with v-Ha-Ras with the normal C-terminal residues and modifications (11). To examine if the decreased membrane association of Ext61L reflected inefficient trafficking or attachment to membranes, the speed with which newly synthesized Ext61L traversed the cytosol and achieved membrane association was assessed. Replicate dishes of cells were metabolically labeled with [35S]methionine/cysteine for 10 min and then one plate was incubated in nonradioactive medium for an additional 30 min. The resulting lysates were separated into soluble (S) and particulate (P) fractions; immunoprecipitates were formed with Ha-Ras antibody; and proteins were resolved by SDS-PAGE. Labeled proteins were detected by fluorography. The culture used to prepare the sample for the 30-min chase point for Ext61L had fewer cells present than the cultures used for the 0-min chase points. The arrowhead designates endogenous Ha-Ras; the arrow indicates the Ext61L protein. B, PC12 cells were plated on serum-coated coverslips and transfected with 1 μg of Ext61L DNA. After 2 days, the cells were fixed and permeabilized and then treated with anti-Ras monoclonal antibody Y13-172 and fluorescein isothiocyanate-conjugated secondary antibody. Cells were viewed by confocal fluorescence microscopy. Scale bar = 40 μm. Display is a representative cell with multiple membrane projections and outgrowth frequently observed in PC12 cells expressing Ext61L. More than 100 cells that possessed neurite extensions were viewed, and all possessed similar plasma membrane-specific distribution of Ext61L. The signal from endogenous Ha-Ras in untransfected cells was too low to be detected (see Fig. 5a for morphology).

intracellular trapping was correlated with poor biological activity. As a more definitive way to assess attachment to specific membranes, immunofluorescence was used to visualize the subcellular localization of Ext61L in intact single cells. A series of confocal laser microscopic images of PC12 cells expressing Ext61L exhibited a clear defined signal of Ext61L at the plasma membrane (Fig. 2B). No staining of internal membranes was observed. These data, taken together with the biochemical data described above, indicated that somewhat less than half of the Ext61L protein succeeded in associating with membranes and, more specifically, that these membrane-bound molecules were located at the plasma membrane.

**Ext61L Associates with Membranes Largely through Hydrophobic Interactions**—The lysines at the terminus of ExtRas were envisioned to participate in ionic interactions with acidic phospholipids of the plasma membrane and thus to initiate membrane binding that would enable palmitoylation. To determine if ionic forces might also contribute to maintenance of the association between the Ext61L protein and cellular membranes, addition of a concentrated salt solution to isolated membranes was used to disrupt ionic interactions (2). Membranes from transfected COS cells were first separated from
lack lipids. Cytosolic (S100) and membrane-containing (P100) fractions were prepared from PC12 cells expressing Ha-Ras61L or Ext61L proteins. The membranes were further partitioned by resuspending the P100 fraction in 1% Triton X-114 and warming the sample to trigger phase separation. For the Ha-Ras61L protein, with the native version of the C terminus, the precursor form (not yet lipid-modified) was present in the cytosol, whereas the mature Ha-Ras61L protein (farnesylated and palmitoylated) appeared in the detergent phase (Fig. 3B). For Ext61L, ~60% of the protein again was cytosolic, and roughly half of the ~40% of the protein that sedimented in the P100 fraction (in multiple experiments, varying from 15 to 25% of the total protein) further partitioned into the detergent phase, suggesting that this minor portion of the expressed protein possessed hydrophobic character (Fig. 3B).

The Ext61L Protein Is Palmitoylated—The two cysteines (Cys-181 and Cys-184) that are palmitoylated in the Ha-Ras61L protein are retained in Ext61L, as is Cys-186, which is normally modified by farnesyl. Thus, the Ext61L protein has three potential sites for palmitate attachment. To determine whether or not Ext61L was palmitoylated, NIH 3T3 cells expressing Ext61L and Ha-Ras61L, respectively, were labeled for 3 h with [3H]palmitate, followed by a chase in nonradioactive medium. At the indicated times, immunoprecipitates were formed, and palmitate labeling of the Ext61L and Ha-Ras61L proteins was detected by fluorography. The Ext61L protein not only incorporated palmitate, but did so at a rate similar to the Ha-Ras61L protein; both required ~3 h for maximum [3H]palmitate labeling (data not shown). During the chase, the depalmitoylation rates for the two proteins were also similar (Fig. 4). When immunoblotting was used to account for variations in Ras protein recovery in the samples on the membrane (data not shown), both Ext61L and Ha-Ras61L lost half of their incorporated palmitate between 2 and 4 h. Similar results were also obtained using PC12 cells transiently expressing either Ext61L or Ha-Ras61L (data not shown). The palmitoylated form of the Ext61L protein was detected only in the membrane-containing cellular fraction, with no evidence of a partially (de)palmitoylated protein being released into the cytosol.2 Thus, although the Ext61L protein was not prenylated, it could be palmitoylated, and the palmitate displayed an apparently normal turnover rate. This indicated that the presence of the polybasic domain did not prevent the Ext61L protein from being accessible to either acylating or deacylating enzymes.

Ext61L Transforms NIH 3T3 Cells—Soluble Ha-Ras61L protein has been reported to act as a dominant-negative inhibitor of Ha-Ras transformation of NIH 3T3 cells (30, 31), presumably by binding and trapping effector proteins, such as the Raf-1 protein kinase, in the cytosol. Because a large amount of

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2 M. A. Booden, unpublished data.
Ext61L was cytosolic, it was possible that the biological effectiveness of Ext61L would be dampened by this pool of cytosolic protein. The ability of the Ext61L protein to stimulate mitogenesis and transformation of NIH 3T3 cells was examined to ascertain if the combination of palmitate and basic residues was capable of supporting a biological function of Ha-Ras. Ext61L DNA was potently transforming and caused robust focus formation (~1100 foci/μg of DNA) (Table I). This activity was equivalent to the transforming activity of DNA encoding the Ha-Ras61L form with the native lipid modifications. The cellular version of the ExtRas protein, ExtWT, did not cause focus formation in NIH 3T3 cells, even when the cells were transfected with up to 2 μg of DNA. This lack of activity was similar to the low transforming activity of the normally lipidated form of Ha-Ras, p21WT. The morphology of transfected NIH 3T3 cells expressing Ext61L was easily distinguished from that of cells expressing the normally lipidated 61L protein: foci produced by 61L were typically swirling, spreading colonies of transformed cells, whereas the Ext61L-induced foci contained clumps of cells that tended to round up and pull away from the plastic substratum and contained unusually long, spindle-shaped, elongated cells with a refractile appearance (data not shown).

To determine if Ext61L expression also triggered anchorage-independent growth, NIH 3T3 cells stably expressing Ha-Ras61L or Ext61L were plated in soft agar and assayed for the ability to form colonies. Like Ha-Ras61L-transformed cells, Ext61L-transformed cells readily formed colonies in soft agar (data not shown). All together, these results demonstrated that the non-prenylated but palmitoylated Ext61L protein produced the aberrant growth properties characteristic of an oncogenic Ha-Ras protein in NIH 3T3 cells.

**Ext61L Causes Neurite Extension in PC12 Cells**—Transformation of NIH 3T3 cells is a hallmark of oncogenic Ha-Ras activity that culminates in focus formation. To assess a different aspect of Ras biological function, the ability of the Ext61L protein to cause differentiation of PC12 cells was examined. In PC12 cells, expression of activated Ras proteins is characterized by the cessation of mitosis, unique rearrangements of the actin cytoskeleton, and extension of neuron-like processes in a program of events similar to those triggered by exposure to nerve growth factor (32, 33). Only activated forms of Ras have been found to produce this response, whereas normal cellular Ras has no effect (34–36). More important, Ras must be membrane-bound to cause neurite extension, as non-lipidated forms, even if activated, are incapable of triggering differentiation (37, 38).

PC12 cells transfected with DNA encoding a cellular, normally lipidated form of Ha-Ras (p21WT) continued to proliferate and showed the limited adherence and round shape characteristic of the parental PC12 cells (Fig. 5, a and b). PC12 cells transfected with DNA encoding the constitutively active, prenylated and palmitoylated Ha-Ras61L protein developed neurites that attained a length of greater than two cell bodies after ~48 h (see Fig. 5 legend). There were two or occasionally three of these axon-like extensions, which continued to elongate over the next 2–3 days. However, at 48 h, only 21% of these extensions were longer than 100 μm (see Fig. 5 legend). The processes were smooth and, during the first days, generally extended linearly, with a single growth cone on each and little branching (Fig. 5e). The amount of DNA necessary to generate this rate and extent of response by Ha-Ras61L was titrated and found to require 1 μg of DNA. The morphology of PC12 cells expressing Ha-Ras61L was similar to that of the cells treated with 50 ng/ml nerve growth factor, although the nerve growth factor response required 2–3 days for neurite extension to be established and progressed more slowly, over 8–10 days (data not shown).

In contrast, PC12 cells transfected with 1 μg of DNA encoding the Ext61L protein developed multitudes of neurites (Fig. 2B). As little as 50 ng of DNA encoding the Ext61L protein caused development of visible neurites as early as 24 h after transfection (Fig. 5f). These processes were longer than those produced by 61L and extended rapidly, exceeding within 2 days even the final length of those in the 61L cultures (Fig. 5e). Cells expressing the Ext61L protein often produced four or five extensions/cell, and 84% of these extensions were longer than 100 μm 48 h after transfection (see Fig. 5 legend). Furthermore, as the differentiation of these cells progressed, the extensions became highly branched, wandered in several directions, and displayed numerous fine filopodia along their length. In addition, Ext61L brought about a flattening of the cell body, with large lamellipodia, areas of adherence, and veils of attachment. A prominent feature of cells expressing the Ext61L protein was the accumulation of numerous large, intracellular vesicles. The biological activity of Ext61L was therefore unexpectedly robust, and the morphological changes it caused were an exaggerated version of those caused by the normally lipidated Ha-Ras61L protein.

To determine if the conspicuous activity of Ext61L could be ascribed to particularly efficient protein expression, the

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**TABLE I**

| DNA transfected | Average No. foci/μg DNA |
|-----------------|-------------------------|
| Ext61L          | 1438                    |
| ExtWT           | 1                       |
| Ha-Ras61L       | 1508                    |
| p21WT           | 2                       |

**Role of Lipids in Ha-Ras Function**

PC12 cells were transfected with the indicated amounts of various Ras DNAs: a, parental cells; b, 10 μg of p21WT; c, 1 μg of Ext61L(C181S/C184S/C186S); d, 1 μg of ExtWT(C181S/C184S/C186S); e, 1 μg of Ha-Ras61L; f, 10 ng of Ext61L; g, 200 ng of ExtWT. Cells were photographed using phase-contrast optics 4 days post-transfection. Scale bars = 50 μm. On day 2, when outgrowths could be clearly assigned to an individual cell body, cell images were captured on computer, and extensions on 50 transfected cells expressing either Ha-Ras61L or Ext61L were measured. A successfully transfected cell was defined as a cell having a flattened and adherent cell body and at least one visible outgrowth. Cotransfections of Ext61L and β-galactosidase expression plasmids showed that >70% of the cells were transfected and that >95% of these transfected cells possessed neurites. Ext61L cells had an average of 3.4 extensions/cell, and 84% of these extensions were longer than 100 μm. For cells expressing Ha-Ras61L, the average number of outgrowths was 2.0, but only 21% of these were longer than 100 μm.
amount of Ext61L or Ha-Ras61L in the membrane/detergent fraction prepared from differentiated PC12 cells transfected as described for Fig. 5 was examined more closely. In the Ext61L-expressing cells, the amount of protein in the detergent fraction was less than the amount present in the detergent fraction of the Ha-Ras61L-expressing cells (Fig. 3B). However, despite expressing smaller amounts of membrane-associated protein, the culture transfected with Ext61L DNA exhibited more extravagant neurite extensions than the parallel culture of cells expressing Ha-Ras61L protein (Fig. 5, e and f). Additional experiments using an even smaller amount of Ext61L DNA (10 ng) showed that exaggerated neurite morphology was still produced when the total amount of Ext61L protein (cytosolic + aqueous + detergent) was much less (approximately one-tenth) than the total amount of protein that was present in the parallel culture transfected with Ha-Ras61L DNA (data not shown). Thus, even with smaller amounts of membrane-bound protein, Ext61L continued to exceed Ha-Ras61L activity and to display unusual characteristics in the neurites it produced. Therefore, in two assays that test quite different outcomes of Ha-Ras activity, transformation and differentiation, there was certainly no evidence that the residues appended to the C terminus or the large amount of cytosolic Ext61L protein diminished biological function.

The Cellular Form of the Extension Protein ExtWT Causes Differentiation of PC12 Cells—The unusual activity of the Ext61L protein in PC12 cells raised the possibility that the cellular (wild-type) form of ExtRas might generate a response in PC12 cells. Transfection of PC12 cells with as little as 250 ng of ExtWT DNA caused production of neurites, with an accelerated time course that was intermediate between that of Ha-Ras61L and Ext61L, and with a morphology characteristic of and as unusual as the Ext61L protein (Fig. 5g). This amount of ExtWT DNA caused production of protein amounts that were less than those produced in parallel cultures expressing Ha-Ras61L protein (data not shown). The quite appreciable activity of the ExtWT protein contrasted with the complete inactivity of normal Ha-Ras with the native C-terminal residues and lipid modifications. Transfection of cultures with 40 times larger amounts of DNA (10 μg) for p21WT produced abundant protein expression (data not shown), but caused no neurite extension (Fig. 5b) (36). Thus, these changes in the Ha-Ras C terminus appeared to have also increased the biological activity of a cellular form of Ha-Ras to cause PC12 cell differentiation.

Palmitoylation Is Required for ExtRas-mediated Differentiation of PC12 Cells and Membrane Localization—Previous observations have established that prenylation and either palmitoylation or a stretch of basic amino acids are required for membrane localization and biological activity of Ras proteins (2, 10). To determine if the exaggerated activity of Ext61L could be ascribed to the polylysine motif, ExtRas proteins that could not be palmitoylated (Ext61L(C181S/C184S/C186S) and ExtWT(C181S/C184S/C186S)) were constructed. The C181S/C184S/C186S mutants were unable to induce differentiation of PC12 cells (Fig. 5, c and d). Thus, the C-terminal polylysine motif, without palmitate, did not support Ha-Ras biological activity.

The subcellular localization of Ext61L and ExtWT(C181S/C184S/C186S) was also examined by subcellular fractionation with Triton X-114 phase separation. As shown in Fig. 3, the ExtRas(C181S/C184S/C186S) proteins, which lack palmitates but retain the lysines, were localized completely to the cytoplasm. Thus, the polylysines alone were not sufficient to maintain association of ExtRas(C181S/C184S/C186S) with the plasma membrane, and stable membrane attachment of ExtRas appeared to require palmitoylation.

**Fig. 6.** Identification of the nucleotide bound to ExtRas proteins. Transfected PC12 cells were radiolabeled with 32P, for 4 h, and Ha-Ras was collected by immunoprecipitation. Protein-associated radioactive GTP and GDP were released, separated by thin-layer chromatography, and detected by autoradiography. The data were quantitated by densitometry, and the ratio of GTP to the total of GTP plus GDP was determined. Lane 1, Ha-Ras61L; lane 2, Ext61L; lane 3, ExtWT.

**Ext61L and ExtWT Bind GTP/GDP Appropriately**—One possible explanation for the pronounced activity of the ExtWT and Ext61L proteins was that the changes at the C terminus altered access of nucleotide exchange proteins or GTPase-activating proteins, which regulate the activity of Ha-Ras to bind GTP and allowed the accumulation of the active form of these proteins, especially ExtWT. To test if the levels of GTP bound to ExtRas proteins were elevated, transfected PC12 cells were labeled with 32P, and radioactive nucleotides bound to Ha-Ras immunoprecipitates were separated by thin-layer chromatography. In differentiated PC12 cells, 83% of the expressed 61L protein and 82% of the Ext61L protein were in the GTP-bound form (Fig. 6). In a second experiment, nucleotides bound to cytosolic and membrane-associated forms of the proteins were analyzed. The portion of the membrane-bound form of Ext61L that had GTP (82%) was the same as the portion that bound GTP in the cytosolic form (data not shown), suggesting that GTPase-activating proteins were equally (in)effective on the Ext61L protein in either location. Thus, both the soluble and membrane-bound forms of Ext61L were, to an extent similar to Ha-Ras61L, predominantly in the active conformation.

For the ExtWT protein, 8% of the protein contained GTP (Fig. 6). This is equivalent to the portion found using this isolation protocol on an overexpressed, cellular Ha-RasWT in NIH 3T3 cell lines, where 8% was also in the GTP-bound form (data not shown). Again, both cytosolic and membrane-associated ExtWT proteins appeared to have similar GTP/GDP ratios (data not shown). Thus, the ExtWT protein was primarily in the GDP state, as appropriate for a cellular form of Ha-Ras, and showed no evidence that the altered C terminus had increased interactions with exchange factors or decreased interactions with GTPase-activating protein(s) to the point that could explain its potent differentiating activity.

**DISCUSSION**

Targeting and stable localization of Ha-Ras to the plasma membrane coincide with acquisition of biological activity. However, the possibility of distinct or independent contributions of the two lipid modifications to targeting, binding, or function has been difficult to examine. We have developed a unique form of Ha-Ras that can be palmitoylated without the requirement for prior farnesylation. The C-terminal changes in this protein do not appear to have altered the basic GTP binding properties of this protein, and the protein causes unexpectedly potent...
biological function of two quite different types. These studies support the possibility that palmitate may have structural and biological roles of its own, independent of isoprenoid.

Requirements for Palmitate Attachment and Removal—To produce a palmitoylated but non-prenylated protein, an artificial polylysine domain was attached to the C terminus of Ha-Ras. The polylysine tail was designed to be (and was apparently successful as) an ionic platform through which the otherwise soluble, non-prenylated Ha-Ras protein could initiate membrane contact and undergo subsequent palmitoylation, which then promoted tighter and more sustained interaction. This tail motif partially mimics the larger polybasic region found just internal to the carboxyl terminus of the Ki-Ras4B protein, which has no cysteines that can be palmitoylated. However, in Ki-Ras4B, the simple presence of a C-terminal polybasic domain, without farnesylation, does not support membrane binding (2, 29, 39, 40).

The ExtRas proteins share several properties with a non-farnesylated mutant yeast Ras2 protein with the C-terminal sequence CCIIKLKRK (mutated CAAX box underlined). This mutant Ras2 protein is also palmitoylated (at the cysteine previously used for farnesyl attachment as well as the natural adjacent site), binds membranes, and is biologically functional (25). Why having a series of positively charged residues beyond the natural C-terminal end of Ha-Ras or Ras2 might permit palmitoylation remains to be studied, but this placement does appear to be a successful method to circumvent the need for prenylation and to permit palmitate attachment and may be applicable to other proteins as well.

The data presented here demonstrate that Ha-Ras can be palmitoylated at the natural C-terminal sites without being farnesylated. Our own studies have indicated that palmitoylation of Ha-Ras, although proposed to be catalyzed enzymatically, has no discernible primary amino acid structure that functions as a potential signal sequence (11). The successful palmitoylation of the non-prenylated Ext61L protein clearly indicates that a farnesyl is not a required part of a recognition motif for any potential Ha-Ras acyltransferases.

A dynamic balance of addition and removal is a central property of Ha-Ras palmitate modification and the characteristic that most clearly differentiates it from prenylation. The Ext61L protein has three cysteines that are potential sites for palmitoylation. Whether all of these sites are used or to what extent each is modified is unknown, as current methods to directly examine palmitate stoichiometry are unsatisfactory. The abundant amount of cytosolic Ext61L protein indicates that palmitoylation of this protein is less complete than that of the native form. The polylysine extension on Ext61L is compatible with dynamic, transient acylation-deacylation and does not prevent access of thioesterases to the C terminus. Future studies can now be designed to remove lysine residues and to replace each cysteine individually to more clearly define favored sites for palmitate attachment as well as the requirements for palmitate removal.

Properties Needed for Membrane Interaction—For Ha-Ras, farnesyl is a rather poor membrane tether: only about 10% of the protein is membrane-bound when a farnesyl is the only lipid present (2, 11). With only palmitate and lysines present, Ext61L interacts with membranes to a greater extent than Ha-Ras(C186S), a property presumably due to the combination of hydrophobic and ionic signals in Ext61L. However, the sizable pool of cytoplasmic Ext61L protein, which has the lysines but no palmitates, suggests that the lysine residues on their own do not contribute greatly to long-term membrane interactions. The solubility of the mutant Ext61L(C181S/C184S/C186S) supports this inference, as does the yeast Ras2 extension protein, where mutation of the cysteines to serines, leaving the polybasic residues intact, abolishes membrane interaction (25). These results argue that firm membrane interaction of an Ha-Ras protein does not require a permanently attached lipid and that it is largely the palmitates, despite their continuous cycles of attachment and removal, that support the stable interaction of the Ext61L protein with membranes. This capacity of palmitates has not previously been demonstrated. Thus, if palmitates can assume responsibility for bilayer interactions, farnesyl does not need to be permanently engaged in this function and becomes available for additional interactions with proteins within the membrane or with effectors. These results emphasize that palmitate and farnesyl may have distinct, perhaps sequential, but complementary roles in membrane binding and function of Ha-Ras.

However, the combination of palmitates and lysines clearly is not as efficient as the >95% achieved by the natural farnesyl plus palmitate modification. One possible explanation for this modest amount of membrane-bound Ext61L protein is that, in the absence of a tethering farnesyl, the dynamic turnover of the palmitates now sets a new, lower equilibrium point for membrane attachment/release. The amount of membrane-bound ExtRas is likely to depend heavily on the rates of palmitoylation and deacylation. The development of the ExtRas proteins should now allow events that may regulate Ha-Ras palmitoylation and membrane interactions to be addressed more clearly.

Other C-terminal alterations in Ha-Ras have produced proteins that are located on intracellular, possibly Golgi, membranes (11). Such data suggest that unexplored determinants of plasma membrane targeting are located in the C terminus of Ha-Ras and may include the sites of palmitoylation. That region remains unaltered in the Ext61L protein and may continue to impart specific plasma membrane interaction signals because immunofluorescence showed strong plasma membrane localization and no staining of internal membranes. Additional studies can now determine if the GTP-bound Ext61L and GDP-bound ExtWT proteins are associated with membrane subdomains (e.g., “rafts” or caveolae) and how their localization compares with that of Ha-Ras protein with the natural lipid modifications. The ExtRas proteins will permit study of the specific roles of palmitate and farnesyl in this compartmentation.

Influence of the Ha-Ras C Terminus on Biological Function—Early studies had suggested that the C-terminal lipids on Ras proteins were only a convenient method for membrane attachment, were not required for more specific interactions with membrane or effector proteins, and were therefore only indirectly involved in signal transduction. This inference was based on studies in which leader sequences (N-myristoylation or an actual transmembrane domain (41)) were used to target non-farnesylated Ha-Ras(C186S) proteins to the plasma membrane. Such chimeric proteins possessed quite good activity in transformation of NIH 3T3 cells, demonstrating that the biological function of transformation did not specifically need farnesyl. However, one group reported that the membrane binding provided by the N-myristoyl group re-established palmitoylation of the two C-terminal Cys-181 and Cys-184 residues (23). That finding remains unconfirmed, as conflicting results have been reported (11), but is potentially important because it implies that acylation may have a direct impact on biological function.

Two additional observations have also implicated the C-terminal lipids in roles beyond membrane anchoring. First, N-myristoylation of a cellular form of non-prenylated Ha-Ras(C186S) potentiated the low transforming activity of the normal protein almost 1000-fold (26). This implies that this
particular N-terminal surrogate membrane targeting system releases cellular Ha-Ras from a restraint that usually keeps such oncogenic activity in check.

Second, some studies have inferred that Ha-Ras lipids, particularly farnesyl, interact directly with proteins, rather than, or in addition to, a membrane bilayer. A farnesyl has recently been reported to be required for interaction of Ha-Ras with a plasma membrane protein (14). A farnesyl-dependent interaction between Ha-Ras and the zinc finger (cysteine-rich domain) of Raf-1 kinase has also been suggested to occur (15, 42). In these instances, the non-farnesylated forms that were used also lacked palmitate, so the contribution or requirement of the acyl group to these interactions remains to be answered. In the yeast Ras2 protein, farnesyl, but not palmitate, is reported to be required for Ras-mediated signal transduction, where it plays an essential, possibly direct role in promoting protein/protein interactions between yeast Ras and a regulatory protein of adenyl cyclase, its downstream effector (20). All these results imply that both C-terminal lipids may have important roles in Ha-Ras effector interaction and function that await exploration.

The strong differentiating and transforming activity of Ext61L was somewhat surprising because previous studies had found that mutant Ras proteins that were poorly membrane-bound were also poorly transforming (10, 11). The potency of Ext61L could not be explained by abnormally stable palmitoylation of the membrane-bound form or by a greater amount of protein in membranes. Even when lesser amounts of membrane-associated protein were present, Ext61L still caused a more rapid and exceptional differentiation of PC12 cells than 61L protein. The morphology of NIH 3T3 cells expressing Ext61L is also distinguishable from that of cells expressing authentic 61L. These unusual morphologies suggest that cytoskeletal pathways are perturbed. Studies are underway to determine if Ext61L interacts with and activates the same cytoskeletal pathways are perturbed. Studies are underway to determine if Ext61L interacts with and activates the same.