Distinct Rates of Palmitate Turnover on Membrane-bound Cellular and Oncogenic H-Ras*

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H-Ras displays dynamic cycles of GTP binding and palmitate turnover. GTP binding is clearly coupled to activation, but whether the palmitoylated COOH terminus participates in signaling, especially when constrained by membrane tethering, is unknown. As a way to compare COOH termini of membrane-bound, lipid-modified H-Ras, palmitate removal rates were measured for various forms of H-Ras in NIH 3T3 cells. Depalmitoylation occurred slowly (t1/2 ~ 2.4 h) in cellular (H-RasWT) or dominant negative (H-Ras17N) forms and more rapidly (t1/2 ~ 1 h) in oncogenic H-Ras61L or H-RasR12,T59. Combining this data with GTP binding measurements, the palmitate half-life of H-Ras in the fully GTP-bound state was estimated to be less than 10 min. Slow palmitate removal from cellular H-Ras was not explained by sequestration in caveolae, as neither cellular nor oncogenic H-Ras showed alignment with caveolin by immunofluorescence. Conversely, although it had faster palmitate removal, oncogenic H-Ras was located in the same fractions as H-RasWT on four types of density gradients, and remained fully membrane-bound. Thus the different rates of deacylation occurred even though oncogenic and cellular H-Ras appeared to be in similar locations. Instead, these results suggest that acylprotein thioesterases access oncogenic H-Ras more easily because the conformation of its COOH terminus against the membrane is altered. This previously undetected difference could help produce distinctive effector interactions and signaling of oncogenic H-Ras.

Mutations causing amino acid substitutions within these Switch domains can produce oncogenic forms of the protein. Although the amino acid changes in oncogenic forms of H-Ras cause a steady-state increase in GTP binding, few structural differences other than in the GTP-binding domains have been noted (3, 4). Thus, current models of H-Ras-mediated oncogenicity envision a heightened signal strength or duration from an otherwise “normal” GTP-bound form of the oncogenic protein.

To produce a biological signal from its interaction with effectors, GTP-bound H-Ras must itself be bound to the plasma membrane. H-Ras acquires membrane association through permanent linkage of a farnesyl isoprenoid and more labile attachment of the fatty acid palmitate to cysteines in its COOH-terminal domain (5–7). Importantly, most structural studies of H-Ras have been performed on soluble proteins that lack these lipids as well as the last ~20 residues of the COOH terminus (8). There is currently no direct information on whether GTP binding might also influence the conformation of the H-Ras COOH-terminal membrane-binding domain. A new approach, biorganic synthesis of lipitated protein (9), holds promise for determining the structure of the C terminus.

One GTP-dependent role of the H-Ras COOH terminus has recently been proposed. This model suggests that inactive forms of H-Ras reside in plasma membrane caveolae and specialized subdomains of the lipid bilayer termed “rafts” (10, 11). GTP binding is then postulated to drive H-Ras from these regions into the general “non-raft” domain of the membrane (12).

Palmitoylation is a clear candidate for a modulator of raft binding, as a growing multitude of acylated proteins are reported to partition into raft-like domains of various kinds (13–15). The presence of palmitate on proteins or membrane targeting sequences has been reported to enhance raft partitioning, whereas its absence often leads to apparently random distribution or decreased membrane binding (15–18). Recently an acyltransferase activity capable of adding palmitate to Go has been identified in raft-like membranes, suggesting that (re)palmitoylation of Go subunit proteins might occur in these subdomains and would support their enrichment there (19). Whether a mammalian homolog of the newly discovered palmitoyltransferase for yeast Ras (20) exists in rafts is not yet known.

Notably, for Go subunits, palmitate attachment and removal has not been linked with lateral movement in the membrane, but rather, with GTP binding, and interaction with regulatory proteins. During stimulation of intact cells with appropriate agonists, palmitates attached to the activated, GTP-bound Go subunits of the Go1, Go4, Go12, and Go13 heterotrimeric G protein families have been reported to show increased turnover (21–25). Additionally, palmitoylation decreases the affinity of Go and Go2 for the RGS (regulator of G protein signaling) proteins.

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that control their rates of GTP hydrolysis, potentially prolonging the lifetime of the active GTP form (26).

Several studies have now provided evidence that the COOH-terminal, lipid-modified domain of H-Ras may influence effector activation. There have been multiple reports that farnesyl-modified forms of H-Ras interact more effectively with, and stimulate Raf-1 kinase more robustly than non-lipidated forms (27–31). Additionally, earlier work had indicated that farnesylation of Ras was important for its interaction with phosphoinositide 3-kinase (32). An H-Ras with a unique palmitoylated but non-farnesylated COOH terminus has been found to hyperstimulate phosphatidylinositol 3-kinase 3-kinase while activating B-Raf kinase poorly (33). Comparisons of H-Ras and K-Ras4B also suggest that the different modes of membrane interaction of these two proteins also produce distinct effector interactions (34). These studies point to the possibility that the lipidated COOH terminus, in addition to its already appreciated role of targeting H-Ras to plasma membranes (without regard for nucleotide state), may also participate in interactions of the GTP-bound form with effectors.

Because of its reversibility and location in the COOH-terminal domain, palmitoylation is a useful reporter for potential structural COOH-terminal differences between cellular and oncogenic forms of H-Ras. Although enzymes are not yet readily available for in vitro manipulation of the palmitoylation state of H-Ras, metabolic labeling and pulse-chase methods with intact cells can be used to measure acylation and deacylation rates of H-Ras. Such in vivo studies should also provide unique information on the COOH terminus while it is attached to the complex and non-homogenous environment of membranes of living cells. We therefore examined the susceptibility to deacylation of cellular and oncogenic forms of H-Ras that displayed large and stable differences in GTP binding. The rates of palmitate removal varied substantially between these proteins, being slower on both a cellular and a dominant-negative form of H-Ras and faster on two oncogenic forms. The faster rates of palmitate removal indicated that, in the oncogenic forms, the palmitoylated Cys-181 and Cys-184 residues of H-Ras were more accessible to palmitoyl thioesterase(s). These results imply that an acylated, membrane-bound COOH terminus of oncogenic H-Ras has a previously undetected difference from cellular H-Ras.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**NIH 3T3 cells stably transfected with Hras61L, H-Ras12R,9T (B. Willumsen, University of Copenhagen), e-H-Ras (L. Quilliam, Indiana University School of Medicine), and inducible H-Ras17N (A. Kralauskas, Harvard Medical School) were grown at 30% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% calf serum (HyClone). The cell line expressing an IPTG-inducible form of H-Ras17N was obtained because long-term expression of this dominant-negative mutant can cause cell death in NIH 3T3 cells. The H-Ras17N mutant had a hemagglutinin epitope tag, so that its apparent size on the SDS-PAGE gel shifted to ~23 kDa. This allowed us to analyze H-Ras17N to be analyzed separately from endogenous H-Ras, which has an apparent size of 21 kDa. The amount of palmitate on endogenous H-Ras was too low to quantify in pulse-chase experiments. The immunoprecipitates were washed, resuspended in special electrophoresis sample buffer with lowered amounts of dithiothreitol and without &-mercaptoethanol (2% SDS, 10 mM NaPO4, pH 7.0, 10% glycerol, 50 mM dithiothreitol, and 0.02% bromphenol blue), resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane and, as needed, sprayed with ENHANCE (PerkinElmer Life Sciences) for fluorography or developed with antibody for immunoblot analysis. Membranes were exposed to film for 3–40 days at ~70°C and the resulting images of palmitoylated H-Ras were quantified using densitometry and ImageQuant (Molecular Biogics).

**Calculations—**As seen previously (38, 39), when plotted on a logarithmic scale, the data did not form a straight line, indicating that a simple exponential decay did not adequately describe the process of palmitate removal (data not shown). The mathematical form that produced the best fit to the data for each of the H-Ras proteins was determined using the Prism GraphPad statistics program. The equation chosen was a two-phase exponential decay line (Y = Span 1-exp(-K1X) + Span 2-exp(-K2X) + plateau), which starts at Span 1 + Span 2 + plateau and decays to plateau with rate constants K1 and K2. The half-life of palmitate reported here is the calculated time that would be needed to remove 50% of the original [3H]palmitate (δt), if the processes were to occur solely at the initial rate, and is equal to 0.693δt, with Span = 0.5.

**GTP/GDP Determination—**Confluent cultures of NIH 3T3 cells were grown overnight in 1% calf serum. Cells were then incubated with 0.5–1 mCi/ml [3H]tritium inorganic phosphate (PerkinElmer Life Sciences) for 4 h in phosphate-free medium containing 1% calf serum (40). H-Ras immunoprecipitates were formed with either monoclonal antibodies 146-3E4 (Quality Biotech) or Y13-259 (Calbiochem). The 146-3E4 antibody was found to isolate H-Ras more efficiently than Y13-259, but the proportions of GTP and GDP were similar with either antibody (data not shown). Labeled nucleotides were eluted from the immunoprecipitates, separated by thin layer chromatography, detected by autoradiography, and quantified using densitometry (41). The percentage of each nucleotide that is GTP (the average of three to four experiments) were calculated by using the equation (% with GTP) = [GTP]/[GTP + GDP] + 1.5 GDP). The GTP content of the 17N protein was not determined because the amount of IPTG-induced 17N protein was small and the anticipated percentages of GTP binding very low.

**Preparation of Subcellular Fractions—**For preparation of soluble and crude membrane-containing fractions, NIH 3T3 cells were lysed in hypotonic buffer (1 mM Tris, pH 7.4, 1 mM MgCl2, 0.1% aprotinin (Calbiochem), with 1 mM Pefabloc, 1 mg/ml leupeptin, 1 mg/ml peptatin (Roche Diagnostics)) by Dounce homogenization. The ionic strength was adjusted to 0.15 M NaCl, and the sample was subjected to centrifugation at 15,000 × g for 30 min. The supernatant (S100) was precipitated with 4 volumes of ice-cold acetone. Equal portions of both the precipitated soluble proteins and particulate fraction (S100) were solubilized in electrophoresis sample buffer, separated by SDS-PAGE on 12.5% gels, transferred to a polyvinylidene difluoride membrane, and selected proteins were detected by immunoblotting.

**Gradients—**Completely detergent-free solubility gradients were based on previous protocols (42, 43), with the following major steps: cells were lysed in the hypotonic buffer as above by passage through a 26-gauge needle, nuclei were removed by low speed centrifugation, then cytosol was removed, and crude membranes were collected by sedimentation at 100,000 × g for 30 min. The P100 pellet was resuspended in gradient buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM dithiothreitol, 10% sucrose, 1% protease inhibitor mixture (Sigma number P-8340)) and homogenized by passage 15 times through a 26-gauge needle. The homogenized sample was then subjected to five rounds of sonication at 15 watt output (model VC 130, Sonics & Materials, Inc.).
3T3 cells were plated in 12-well culture cassettes on glass coverslips (18 luminescence (Pierce 34080). (anti-mouse or anti-rabbit, Pierce) were used with detection by chemiluminescence as above, then immediate neutralization by addition of 1/3 volume of 0.5 M MES buffer (pH 5.0), and confirming that the solution had been adjusted to pH 7.5. Detergent-treated samples were prepared starting from the P100 as above, but pretreatment consisted of resuspending the P100 in gradient buffer containing 1 or 0.1% Triton X-100, on ice, for 30 min. Gradients were prepared as above, with Triton-treated samples were overlain with Optiprep gradient buffer, without Triton (15, 44).

**Immunoblotting**—Proteins collected by trichloroacetic acid, acetone precipitation, or immunoprecipitation were resuspended in electrophoresis sample buffer. After separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes and nonspecific protein binding was blocked by incubating the membrane in 1.25% nonfat dry milk in Tris-buffered saline overnight. Membranes were probed with the H-Ras-specific mouse monoclonal antibody, 146-3E4 (Quality Biotech), a rabbit antibody to caveolin-1 (C19630, Transduction Laboratories), or mouse antibody to the transferrin receptor (Zymed Labs). For [3H]palmitate-labeled samples, biotinylated secondary antibodies (anti-mouse or anti-rabbit, Vector Laboratories) were used with detection by alkaline phosphatase (Vector Laboratories) using the manufacturer’s protocol. The amounts of radioactive palmitate bound to the H-Ras protein were quantified along with the amount of H-Ras protein in each sample to correct each fluorographic signal for variations in protein recovery in the immunoprecipitates. For density gradient samples, horseradish peroxidase-labeled secondary antibodies (anti-mouse or anti-rabbit, Pierce) were used with detection by chemiluminescence (Pierce 34080).

**Sonication and Immunofluorescence of Membrane Fragments—**NIH 3T3 cells were plated in 12-well culture cassettes on glass coverslips (18 mm) coated with 100 µg/ml poly-t-lysine (Sigma) and 50 µg/ml fibronectin (Sigma). Two days later the cells were processed, on ice, as described (44, 45) in a sonication buffer that contained GDP/MgCl₂, with the sonication probe placed 12 mm above the coverslip and sonicated for a 1-s burst at 4 watts using the Sonics Vibra Cell ultrasonic processor and washed in ice-cold sonication buffer. Samples were fixed with fresh 4% formaldehyde on ice and quenched with 50 mM ammonium chloride, then incubated with blocking buffer (2% horse serum and 0.4% bovine serum albumin in phosphate-buffered saline). Sonicated cells were incubated for 1 h at room temperature with anti-H-Ras (Y13-238, 1:500 dilution, 0.5 µg/ml) and anti-caveolin (C19630, 1:4000 dilution, 0.625 µg/ml, Transduction Laboratories) diluted in the blocking buffer. Secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG from Molecular Probes) were diluted 1:700 in blocking buffer, to 2.8 µg/ml. The slips were mounted in Vectashield mounting medium (Vector Laboratories) and photographed using a x100 oil immersion lens on a Nikon Eclipse E800 microscope with Spot RT digital camera (Roche Diagnostics). Images were captured in color, imported into and overlaid using Adobe Photoshop. The alignment control was incubated as above using the polyclonal anti-caveolin antibody and both a green Alexa Fluor 488 goat anti-rabbit serum and red Alexa Fluor 594 goat anti-rabbit serum and showed 100% alignment (data not shown).

**RESULTS**

**Palmitate Removal on Oncogenic H-Ras Is Faster Than on Cellular H-Ras**—We undertook a detailed examination of H-Ras palmitate labeling, turnover, and GDP binding in NIH 3T3 cells, a cell line in which effector pathway connections and consequences for transforming activity of H-Ras were well studied. A pulse-chase technique was used to examine the rates of palmitate removal/replacement in NIH 3T3 cell lines expressing the cellular (WT) form of H-Ras or the activated, oncogenic 61L protein. Cells were labeled for 3 h with [3H]palmitate, then incubated for the indicated times in medium containing non-radioactive palmitate. As palmitate addition is a post-translational modification, cycloheximide can be included in the medium, to prevent synthesis of new protein that might become palmitoylated during the experiment. Thus only removal of palmitate from previously synthesized and palmitoylated H-Ras was detected during the chase. Results of these pulse-chase experiments showed that the 61L protein lost [3H]palmitate rapidly, with a decrease to less than half of its initial value within 1 h (Fig. 1). In contrast, palmitate on the WT protein was removed more slowly, and still retained almost all of its initial palmitate content after 2 h (Fig. 1).

To determine whether another oncogenic form of H-Ras would display a similar rapid rate of palmitate removal, cells expressing a H-Ras12R,59T (v-H-Ras) protein were tested (Fig. 1). This second activated protein also displayed a rapid decrease in the amount of radioactive palmitate, which remained attached, with more than half of the [3H]palmitate being lost in 2 h.

To test if a second H-Ras protein that would be largely GDP-bound, similar to the cellular H-Ras WT, might show a slow rate of palmitate removal, a 17N mutant of H-Ras was analyzed. The S17N point mutation causes the protein to accumulate in the inactive GDP-bound form (35). Over the time course of the label and chase, no changes in cell morphology or cell death in the induced H-Ras17N cultures were observed (data not shown). Experiments performed on IPTG-treated NIH 3T3 cells expressing this H-Ras17N indicated that palmitate on the GDP-bound 17N mutant was removed slowly, with very little [3H]palmitate signal being removed after 2 h (Fig. 1). Thus, two oncogenic forms of H-Ras had faster rates of palmitate removal and two predominantly GDP-bound forms of H-Ras had slower rates of removal.

Because H-Ras has two sites of palmitoylation and only a small amount of palmitate was removed in the short pulse-chase experiments, the chase period was extended to 24 h. The results showed, as had been seen previously (38, 39), that >85% of the [3H]palmitate could be removed, indicating that both Cys-181 and Cys-184 sites were susceptible to deacylation (Fig. 2). However, there were clearly two phases of decrease in the [3H]palmitate. A fast rate was most visible during the first

**FIG. 1.** Palmitate removal from oncogenic H-Ras occurs more rapidly than from cellular H-Ras WT or H-Ras 17N. NIH 3T3 cells expressing H-Ras61L, H-Ras12R,59T, the cellular form H-Ras WT, or an IPTG-induced H-Ras17N were incubated for 3 h with [3H]palmitate, then incubated for the indicated times (Chase) in non-radioactive medium containing 200 µM non-radioactive palmitate. H-Ras immunoprecipitates were formed and separated by SDS-PAGE and [3H]-labeled H-Ras was detected by fluorography.

![Image](http://www.jbc.org/)

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30 min, whereas a slow rate overlapped with the first and continued during the remainder of the chase (Fig. 2). Because the half-life of H-Ras polypeptides in growing cells is ~24 h (37), the degradation of each form of H-Ras during the extended chase was monitored by immunoblotting. No significant differences in stability were noted between the cellular and oncogenic forms (data not shown). However, the amount of H-Ras protein in each sample was quantified to correct each fluorographic signal for variations in protein amount and recovery in the immunoprecipitates.

Calculation of Initial Rates of Palmitate Removal on H-Ras Proteins—When plotted on a logarithmic scale, the data from these experiments did not form a straight line (data not shown). This indicated that a simple monoeponential decay model could not be used to adequately describe the process of palmitate removal. Similar results showing two phases of decrease in [3H]palmitate have been reported previously (39). Palmitate removal on the singly palmitoylated N-Ras also fails to produce a linear line on a log plot (46). This may result from removal of [3H]palmitates and replacement at the longer time points with others of lower specific activity. The overlap of the two phases of [3H]palmitate decrease made simple linear extrapolation unusable for estimating the rates of palmitate loss. Therefore, to determine the palmitate half-life, the data were analyzed mathematically. The calculated rates for the first phase of palmitate removal indicated again that palmitate removal was more rapid in the two oncogenic forms of H-Ras than in the cellular or 17N versions (Table I). For the oncogenic H-Ras61L and H-Ras12R,59T proteins, the more rapid phase occurred with a $t_{1/2}$ of ~1 h. This rate was more than twice as fast as that of the WT and H-Ras17N proteins, which had $t_{1/2}$ values of longer than 2 h.

![Graph](image)

**Fig. 2.** H-Ras palmitate removal can be described by a two-phase exponential decay model. Data from multiple (4–7) pulse-chase experiments were compiled and analyzed as described under “Experimental Procedures.” Because the polypeptide chain of H-Ras has a half-life of ~24 h, immunoblotting was used to correct [3H]palmitate values for the amount of protein remaining. Data points obtained from the same times of chase are displayed as the average ± S.E. The lines represent the palmitate decrease calculated using the two-phase exponential decay model. Note that the graphs have linear y axes, not logarithmic.

| H-Ras protein | Palmitate half-life$^a$ (h) | % in GTP form |
|---------------|----------------------------|--------------|
| H-RasWT       | 2.4 (2.1–2.9)              | 12           |
| H-Ras17N      | 2.2 (1.9–2.4)              | ND$^b$       |
| H-Ras12R,59T  | 0.9 (0.7–1.2)              | 61           |
| H-Ras61L      | 1.0 (0.8–1.4)              | 72           |
| H-Ras GDP     | Calculated$^c$             | Assigned     |
| H-Ras GTP     | 0.12                       | 100          |

$^a$The half-life is the time needed to remove 50% of the original palmitate if the decrease in [3H]-signal were to occur solely at the faster rate.

$^b$Values are calculated with the two-phase exponential decay model using data from three to six separate experiments. Numbers in parentheses are the range of values calculated from $k_1$ (± S.E.). Half-life = 0.693$/k_1$.

$^c$Values are the averages from three to four separate experiments. ND, not determined.

$^d$Values are calculated from the equation of the line in Fig. 3B.

**Table I.** Palmitate removal rates and GTP binding of cellular and oncogenic H-Ras

**Determination of Portion of Different H-Ras Proteins That Bind GTP**—The rate of removal of the [3H]palmitate did not correlate with growth rates of the different cell lines, as the WT and 61L cells had similar doubling times of ~18 h. Also, there was not a good correlation with the amount of protein expressed, because the WT cells expressed the largest amount of protein, the 17N cells the least, and the H-Ras12R,59T proteins intermediate levels. The pattern of palmitate removal rates appeared to correlate best with the nucleotide binding properties expected for the different H-Ras proteins, with the oncogenic proteins, in which a larger portion of the protein should be in the GTP form, showing the fastest decylation.

The amount of each protein that was present in the GDP or GTP form was determined directly (Table I). The significant amount (39%) of GDP-bound H-Ras12R,59T emphasized that even in cells expressing oncogenic H-Ras, the palmitate removal rates had been determined from a mixed population of both GDP- and GTP-bound H-Ras proteins. The palmitate removal rates and GTP binding data for each protein were therefore used to estimate what the rates of palmitate removal might be if all of the protein was entirely in either the GTP- or the GDP-bound state (Fig. 3). This calculation predicted a palmitate half-life of ~7 min for a GTP-bound protein and palmitate half-life of nearly 2.7 h for a GDP-bound H-Ras protein: a 25-fold increase in rate of removal of palmitate upon GTP binding (Table I). If the H-Ras61L protein were to have a 1.4-h palmitate half-life, the $t_{1/2}$ of palmitate on the GTP-bound form might be as long as 28 min, but would still be 5 times faster than the removal rate on GDP-bound H-Ras. It is important to stress that these calculations are based on the assumptions that palmitate removal rates are affected only by conformational changes occurring upon GDP or GTP binding, and that there is a linear relationship between these properties. Both of these assumptions are almost certainly oversimplifications that will be improved with further study. Even these rudimentary estimates revealed that differences in palmitate turnover rates between GTP- and GDP-bound forms of H-Ras could be substantial.

**Comparison of [3H]Palmitate Incorporation into Cellular and Oncogenic H-Ras**—In labeling experiments, the amount of [3H]palmitate found in a protein represents the balance between the rates of addition and removal during the incubation period. To learn if oncogenic proteins also showed a change in palmitate attachment, the rates of incorporation of [3H]palmitate into the cellular or H-Ras12R,59T proteins were compared. A faster rate of palmitate removal should produce more sites...
that could be acylated, and this could result in enhanced labeling of oncogenic H-Ras with \[^{3H}\text{palmitate, if removal is the rate-limiting step and the rate of palmitate attachment remains the same. To focus only on mature proteins, cycloheximide was included in the labeling medium to prevent \[^{3H}\text{palmitate incorporation into newly synthesized proteins. Using this protocol, both WT and H-Ras12R,59T became labeled to 50% of their final, steady-state extent of palmitate incorporation in less than 1 h and to >95% within 2 h (Fig. 4, A and B). This confirmed that 4 h was an appropriate point for initiating the chase experiments, as labeling of both proteins was steady by that time. Although the portion of labeled H-Ras12R,59T occasionally appeared to lag a bit behind that of the WT protein, given the variations inherent with such rapid rates of incorporation the measurements were not significantly different. The \[^{3H}\text{palmitate signal of both WT and H-Ras12R,59T often decreased slightly after 4 h. A similar modest decrease in labeling after an initial plateau has been observed in other proteins, including the \(\beta\)-adrenergic receptor (47). Of particular note, \[^{3H}\text{palmitate labeling of oncogenic H-Ras occurred with a rate roughly similar to cellular H-Ras. To maintain an equivalent amount of attached \[^{3H}\text{palmitate, given that more rapid palmitate removal from oncogenic H-Ras continued to occur throughout the labeling period, this implied that palmitoylation of the oncogenic protein may also be accelerated. This possibility will require further study, likely using \textit{in vitro} techniques, as the early time points of metabolic labeling experiments do not reflect steady-state conditions because \[^{3H}\text{palmitate is also utilized for many additional pathways. Thus, both the labeling and pulse-chase data suggest that palmitate turnover on oncogenic forms of H-Ras is enhanced.}

\textit{H-RasWT Does Not Co-align with Caveolin in Native Membranes—The mechanisms that regulate palmitate removal on H-Ras proteins are almost completely unknown. One possibility was that decreased removal might occur if H-RasWT were to reside largely within the physical structure of caveolae, where the protein might be sheltered from deacylases. Although there have been reports that H-Ras can be found within caveolae in baby hamster kidney cells or Madin-Darby canine kidney, using immunogold electron microscopy (12, 48), we wished to observe the distribution of caveolin with H-RasWT and oncogenic H-Ras proteins in NIH 3T3 fibroblast cells on a larger scale, yet without permeabilizing the membranes with solvents or detergents. For this we adopted a technique used previously to study the localization of G protein subunits and the related GTPase, TC10, in which sonication of cells grown on coverslips is used to remove the overlying cell body and reveal the basal plasma membrane (44, 45, 49–52). The adherent plasma membrane is then fixed with formaldehyde and proteins are detected \textit{in situ} without exposing the membrane fragments to detergent. This procedure for observation of proteins in native membranes showed caveolin staining as small dots, presumably representing its location in caveolae on the basal surface of the NIH 3T3 fibroblasts (Fig. 5). H-RasWT was also present as numerous small dots on the basal plasma membrane (Fig. 5). However, when observed at higher magnification, there was very little overlap between the dots of H-RasWT and those of caveolin (Fig. 5, merge), although both proteins were intimately intermingled. For caveolin, some of the dots aligned with H-RasWT dots and appeared yellow in the merged image. However, many caveolin puncta had no apparent association with any H-RasWT structure and remained red. More importantly, there were more spots of H-RasWT, so most of the \textit{green} H-RasWT dots had no caveolin counterparts. Likewise, on membranes prepared from cells transformed by H-Ras61L, only a small portion of overlap between the oncogenic H-Ras and caveolin was observed, and most of the H-Ras61L was outside of the caveolin-containing puncta (Fig. 5). H-Ras12R,T59T also showed little alignment with caveolin (data not shown). Importantly, because the majority of H-RasWT was located outside caveolae, there was no indication that a caveolar refuge from deacylases would explain the slow rate of H-RasWT depalmitoylation.}

\textit{Equivalent Distribution of Oncogenic and Cellular H-Ras in Membrane Domains—Several studies have now suggested that H-Ras proteins may partition into specialized cholesterol- and sphingolipid-rich membrane microdomains, popularly termed rafts (12, 53). Although only a small amount of H-RasWT and H-Ras61L appeared to reside directly within the caveolar subset of rafts, the distributions of cellular and oncogenic forms of

\textit{Rapid Palmitate Turnover in Oncogenic H-Ras

Fig. 3. Correlation of GTP and GDP binding with palmitate removal rates in cellular and oncogenic H-Ras proteins. The palmitate half-life for each protein calculated from direct measurements (shown in Table I) was graphed versus the measured percent GTP bound. The equation of the resulting line (inset) was used to calculate the theoretical palmitate half-lives of H-Ras that was fully in the GDP-bound (0% GTP) or GTP-bound (100% GTP) form, and is shown in Table I.

Fig. 4. Palmitate is incorporated into WT and H-Ras12R,59T at similar rates. NIH 3T3 cells expressing \(\alpha\) H-RasWT or H-Ras12R,59T were labeled with \[^{3H}\text{palmitate for the indicated times. H-Ras was immunoprecipitated, proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and exposed to film for 9–30 days. The upper, phosphorylated form of H-Ras12R,59T is indicated. B, H-Ras protein on the membranes was detected by immunoblotting and \[^{3H}\text{palmitate values corrected for amount of protein and graphed. The data points are an average of three to four separate experiments.}
Fig. 5. **H-RasWT and H-Ras61L co-align poorly with caveolin in native membranes.** Adherent membrane fragments from sonicated NIH 3T3 cells expressing H-RasWT or H-Ras61L were fixed with formaldehyde and then stained directly with antibodies to H-Ras and caveolin with appropriate secondary antibodies labeled with fluorescent probes. Images were captured in color using a ×100 oil immersion objective (scale bar = 10 µm), imported into Adobe Photoshop, and merged.

H-Ras were further compared using multiple biochemical fractionation techniques.

One prevalent method of raft membrane preparation exposes membranes to 0.15 M (occasionally even 0.5 M) sodium carbonate (pH 11), followed by sonication to disrupt cytoskeletal attachments to membrane fragments (12, 54, 55). This technique is risky for H-Ras proteins, as the thioester bonds between the palmitates and protein are susceptible to high pH buffer, and quite substantial amounts of MES buffer must be used to thoroughly neutralize the carbonate. As has been noted by others (56), for K-Ras4B proteins, which rely upon a highly basic, lysine-rich domain for attachment to membranes, pH 11 will neutralize the positive charge of the lysine and potentially allow release or new, inappropriate partitioning of the protein. Using this non-detergent method, with immediate, careful neutralization of the carbonate and thorough sonication (see "Experimental Procedures"), caveolin-containing membranes could be separated, as expected, in the top fractions of the gradient (Fig. 6A). As seen previously (12, 43, 57), the H-RasWT protein was also present in these fractions (Fig. 6A).

Similarly, the H-Ras61L protein was found primarily in buoyant membranes (Fig. 6A).

In light of the concerns noted above, a neutral pH, completely detergent-free gradient separation has also been adopted in some studies (42, 58). Because cytoskeletal structures are known to differ between normal and transformed cells, multiple, brief rounds of sonication (19) were used to ensure complete disruption of cytoskeletal structures that might ensnare H-Ras and prevent its flotation. After removal of the much more abundant cytosolic proteins, a profile of the crude P100 membrane proteins distributed in the different fractions showed that a very abundant, ~44-kDa protein (presumably actin) remained largely at the bottom of the gradient (Fig. 6B).

Although early models envisioned that what would now be called rafts might occupy only 10% of the membrane, the results here are in line with studies that now estimate raft-like microdomains may cover over one-third of the cell surface (59, 60). In these neutral gradients, H-RasWT was again found in membranes at the top of the gradient, as was caveolin (Fig. 6C).

In addition, essentially all of H-Ras12R,59T was present on membranes in the upper fractions of the gradient (Fig. 6C; also H-Ras61L, data not shown). These results from both carbonate and neutral pH gradients agree with those reported recently for cellular and H-RasV12 (58).

A second method for examining association of a protein with membrane subdomains takes advantage of the tight lipid packing in these regions, which causes the domains to resist dissolution in cold, non-ionic detergents, and allows their separation as detergent-resistant membranes (DRMs). In membranes exposed to a standard, ice-cold 1% Triton X-100 extraction protocol, we found, as have others (15, 61, 62), that H-Ras, whether cellular or oncogenic (Fig. 6D), was absent from DRMs. The 1% Triton X-100 also extracted some of the caveolin (Fig. 6D).

Other laboratories have found that some proteins, notably the T cell receptor and the FcεR1 receptor of mast cells, are released by exposure to 1% Triton X-100, but can be successfully isolated with DRMs if the amount of Triton X-100 is decreased to less than 0.2% (63, 64). To examine if H-Ras proteins were also in this category, samples were pre-treated with only 0.1% Triton X-100 and membranes were separated on detergent-free gradients. In these gradients, caveolin-containing DRMs floated to the top of the density gradient (Fig. 6D).

Transferrin receptor, a marker for non-raft, detergent-sensitive domains, was found in the bottom fractions of the gradient, as expected. However, H-RasWT and H-Ras61L (Fig. 6D) were again separated from the DRMs. This indicated that in NIH 3T3 cell membranes the interactions of both types of H-Ras were sensitive to even low amounts of Triton X-100.

Thus, on four types of density gradients, the properties of membranes containing cellular or oncogenic H-Ras were indistinguishable. In the absence of detergent, both proteins appeared to reside almost completely in buoyant membranes of a density similar to that of caveolae. If samples were exposed to Triton X-100, both cellular and oncogenic proteins were separated from these membrane subdomains. Consequently, despite the more rapid palmitate removal on oncogenic H-Ras, there was no significant difference in its subdomain partitioning in NIH 3T3 cells compared with the cellular protein.

**Overall Membrane Binding of Oncogenic and Cellular H-Ras Is Similar**—An alternative possible outcome of the more rapid rate of depalmitoylation of oncogenic H-Ras might be an accumulation of non-palmitoylated protein. Determining the acylation stoichiometry of proteins has been notoriously difficult, given the lability of thioester-linked palmitoyl groups during sample preparation. However, because a non-palmitoylated C181S/C184S mutant H-Ras protein is ~90% cytosolic (37) it appeared that even a modest pool of completely non-acylated protein might be detectable using simple fractionation techniques. NIH 3T3 cells expressing WT, H-Ras12R,59T, or H-Ras61L were separated into cytosolic (S100) and particulate (P100) fractions. Results of three experiments for each H-Ras protein showed that cellular and oncogenic proteins were membrane-bound to similar extents, with <10% of the proteins in the soluble S100 fraction (Fig. 6E). This result indicates that rapid depalmitoylation does not lead to an accumulation of oncogenic H-Ras in the cytosol. Indeed, the apparent balance of palmitoylation and depalmitoylation and the similarity in membrane binding may indicate that the stoichiometry of palmitoylation is similar between cellular and oncogenic H-Ras. If in vitro assays become possible, this indirect estimation of palmitate content will need to be examined more accurately, to learn if transient depalmitoylation can release H-Ras or if,
Once H-Ras interacts with the membrane, it can be retained by other means. Whatever the mechanisms that govern membrane interaction, there appears to be little difference in either specific or general membrane binding between the cellular and oncogenic forms of H-Ras.

**DISCUSSION**

**Oncogenic H-Ras Is Depalmitoylated More Rapidly Than Cellular H-Ras**—There have been few comparisons of the rates of palmitate removal for cellular and oncogenic forms of H-Ras. The current studies show, unexpectedly, that two oncogenic forms of H-Ras have faster rates of depalmitoylation than does cellular H-Ras or a GDP-bound form of the protein. This faster rate of depalmitoylation of oncogenic H-Ras indicates that the palmitates of oncogenic H-Ras are more susceptible to attack by cellular acylprotein thioesterases. The primary candidate for such an enzyme is the acylprotein thioesterase, APT1, although it has so far been shown to deacetyl H-Ras only in vitro (65, 66). The labeling experiments, although less definitive, suggest that oncogenic proteins may also be more accessible to a palmitoyl transferase. The Erf2/Erf4 complex, the first enzyme known to palmitoylate a (yeast) Ras protein, is located primarily in the endoplasmic reticulum (67). Whether a similar enzyme in mammalian cells is located at the plasma membrane, where steady-state (re)palmitylation is currently thought to occur, is not yet known.

The different rates of deacylation occurred even though oncogenic and cellular H-Ras appeared to be in similar locations. Oncogenic proteins displayed biochemical properties of membrane binding that were comparable with that of the cellular H-Ras. By high-resolution immunofluorescence, neither cellular nor oncogenic proteins could claim shelter in caveolae, as the great majority of each protein was located outside of these specialized domains. There are now reports that H-Ras is also present in early endosomal structures (68, 69). However, a recent study suggests that this localization is also independent of the GTP status of H-Ras (70). Therefore, escape from deacylases through differential endosomal trafficking may not explain the differences in cellular and oncogenic H-Ras deacetylation. Overall, it was not apparent that thioesterases would encounter any physical limitation (or preference) in access to either cellular or oncogenic H-Ras.

The various gradient separations used here and in the literature are subject to conflicting interpretations. Non-detergent methods have no intrinsic features that should discriminate the density of raft and non-raft domains from plasma membranes. They also rely on thorough and reproducible sonication to disrupt cytoskeletal structures that will vary with cell type. Detergent-based methods must rigorously maintain consistent ratios of detergent to cellular lipids. Using the detergent-based approach, there are compelling studies that indicate prenylated proteins in general, and H-Ras specifically, are not in raft-like membranes (15, 71). However, there are also reports that the biophysical "impairment" of an isoprenoid does not exclude active Ras proteins from raft-like membranes in living cells (72). At an even more fundamental level, there still are no guidelines to distinguish a protein that is loosely raft-embedded and easily dislodged by detergent, from a protein in a disordered membrane that becomes dissolved by the detergent. The important conclusion for H-Ras palmitoylation is that we and others find cellular and oncogenic forms in locations that are indistinguishable, and there is no sign that thioesterases will encounter oncogenic H-Ras proteins in a more exposed position, or cellular H-Ras in a more protected site.

**Implications of Different COOH-terminal Conformations in Oncogenic and Cellular H-Ras**—With evidence from multiple
approaches and studies indicating that the majority of cellular and oncogenic forms of H-Ras are in similar locations, it appears possible that the vulnerability of oncogenic H-Ras to thioesterases arises because its acylated COOH-terminal region is juxtaposed to the membrane in a distinct way. No conformational information of the native COOH-terminal domains of oncogenic and cellular H-Ras is available from previous structural studies. The current work offers significant information because the results are derived from full-length H-Ras proteins that are acylated and constrained by bilayer association. It also suggests that palmitoylation should be a useful reporter for conformational studies of the COOH terminus of H-Ras as it interacts with membranes.

The results provided here suggest that structural alterations in the COOH-terminal domain are present in oncogenic forms of H-Ras. This model expands and complements current visions of how the COOH terminus may participate in H-Ras signaling. Such changes could arise from transmission of the effects of the oncogenic mutation itself, or from the GTP binding the mutation induces. There is some theoretical data to suggest that the H-Ras membrane-binding domain may be in a position to respond to conformational changes in the Switch I and II regions that occur upon oncogenic mutation. Brandt-Rauf et al. (73) used conformational energy analysis to predict the structure of the last 18 amino acids of the H-Ras carboxyl terminus and integrated their predicted carboxyl terminus with the known crystal structure of the remainder of the protein. Although that work did not include acyl groups, the results showed a low energy conformation in which the COOH-terminal domain was near the amino terminus and Switch II region of H-Ras. Thus, the work here supports the possibility that the H-Ras COOH terminus could be an unexplored partner to the better studied Switch domains. A similar model has been proposed from the three-dimensional structure of the related GTPase, cdc42. This structure provides the first look at a full-length COOH-terminal domain of a small GTPase (although still lacking lipids) and suggests that the isoprenoid-modified cysteine of cdc42 will be juxtaposed to the Switch II domain (74).

The data here also support and expand the increasingly attractive possibility that the COOH-terminal region may represent an additional surface through which regulatory or effector proteins may bind to H-Ras. For instance, proteins responsible for trafficking H-Ras to the cell surface may detect the COOH-terminal lipid modifications (75). For the effector protein Raf-1 kinase, selective interaction with H-Ras and K-Ras COOH-terminal lipid modifications (75). For the effector protein Raf-1 kinase, selective interaction with H-Ras and K-Ras COOH-terminal structure might contribute to different signaling outcomes for cellular and oncogenic H-Ras. Although oncogenic H-Ras proteins remain attached to membranes, an interesting prospect raised by a more active participation of the COOH terminus in H-Ras signaling is the possibility that palmitoylation may not be the only mechanism that sustains H-Ras membrane binding.

It is notable that this COOH-terminal conformation would occur only in forms of the oncogenic protein that are tethered to the membrane, and thus would no longer be present in soluble oncogenic protein in cells treated with farnesyl transferase inhibitors (78). The results here give a firmer basis and even greater emphasis to the promise of drugs that may disable oncogenic Ras activity through control of lipids. This work also suggests that targeting the acyl modification may have an impact on Ras signaling that is as profound as that from inhibiting farnesylation (79–81).

Does (De)palmitoylation of H-Ras Play a Role in Signal Transduction?—From our observations and those of others, the rate of palmitate removal on cellular H-Ras is on the order of hours (38, 39). This depalmitoylation rate is clearly too slow to participate directly in rapid signal transduction events, and gives the impression of a basal, constitutive renewal of acyl groups that is more likely to be influenced by the general availability of acyl-CoA in the cell. It is intriguing that the more rapid acyl removal calculated for GTP-bound H-Ras occurs in a time frame fast enough to suggest that palmitates could participate in (probably the later stages of) signaling events. Such a possibility has been suggested for the Gα subunit where the agonist-stimulated turnover of palmitate can be as fast as 2 min (82). It will be interesting to determine in more detail how palmitate turnover, COOH terminus conformation, and possibly microdomain residence are inter-related. The possibility that COOH-terminal changes can report and perhaps participate in the active state emphasize the importance of developing new methods to study fully lipid-modified, membrane-bound H-Ras.

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