S-Acylation and Plasma Membrane Targeting of the Farnesylated Carboxyl-Terminal Peptide of N-*ras* in Mammalian Fibroblasts[†]

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ABSTRACT: We have used a series of fluorescent lipid-modified peptides, based on the farnesylated C-terminal sequence of mature N-*ras* [-GCMGLPC(farnesyl)-OCH_3], to investigate the membrane-anchoring properties of this region of the protein and its reversible modification by S-acylation in cultured mammalian fibroblasts. The farnesylated peptide associates with lipid bilayers (large unilamellar phospholipid vesicles) with high affinity but in a rapidly reversible manner. Additional S-palmitoylation of the peptide suppresses its ability to desorb from, and hence to diffuse between, lipid bilayers on physiologically significant time scales. NBD-labeled derivatives of the farnesylated N-*ras* C-terminal heptapeptide, when incubated with CV-1 cells in culture, are taken up by the cells and reversibly S-acylated in a manner similar to that observed previously for the parent protein. The S-acylation process is highly specific for modification of a cysteine rather than a serine residue but tolerates replacement of the peptide-linked farnesyl moiety by other hydrophobic groups. Fluorescence microscopy reveals that in CV-1 cells the S-acylated form of the peptide is localized preferentially to the plasma membrane, as has been observed for N-*ras* itself. This plasma membrane localization is unaffected by either reduced temperature (15 °C) or exposure to brefeldin A, treatments which inhibit various trafficking steps within the secretory pathway. These results suggest that in mammalian cells the plasma membrane localization of mature N-*ras* is maintained by a 'kinetic trapping' mechanism based on S-acylation of the protein at the level of the plasma membrane itself.

A number of proteins involved in cellular regulation carry a single permanent lipid modification (a myristoyl or prenyl group) and in addition can be modified by reversible S-acylation on a cysteine residue near the site of the first lipid modification. The S-acylation of such proteins is important for their proper subcellular targetting and their physiological function [for reviews, see Resh (1994), Casey (1995), Jackson et al. (1995), Milligan et al. (1995a,b), Ross (1995), and Bhatnagar and Gordon (1997)].

Like other *ras* isoforms, the mature form of N-*ras* is targeted to the plasma membrane of mammalian cells (Grand et al., 1987). Plasma membrane targeting of these proteins is conferred by a farnesylated/O-methylated C-terminus accompanied by either an adjacent polybasic sequence (K-*ras*4B) or one or more sites of S-acylation (K-*ras*4A, H-*ras*, and N-*ras*). For N-*ras*, the protein-coupled *S*-acyl group has been shown to exhibit rapid turnover (Magee et al., 1987). S-Acylation has been shown to be important for the correct subcellular targeting of H-*ras* (Hancock et al., 1989, 1990, 1991; Willumsen et al., 1996), and appears likely to play an important role in the targeting of the related N-*ras* as well. However, at present the nature and subcellular localization of this S-acylation reaction are unclear. Apparently enzymic activities mediating *in vitro* S-acylation of farnesylated (but not of unmodified) N-*ras*, or of a C-terminal peptide of N-*ras*, have been reported (Gutierrez & Magee, 1991; Liu et al., 1996) and in one study localized to a subcellular fraction tentatively identified as a Golgi subcompartment (Gutierrez & Magee, 1991). This latter result could suggest that mature N-*ras* is S-acylated at the level of the Golgi apparatus and subsequently transferred to the plasma membrane by vesicular membrane transport. By contrast, recent evidence suggests that certain other palmitoylated[1] proteins, including the α-subunits of various G-proteins and the nonreceptor tyrosine kinase $p59^{fyn}$, may be S-acylated directly at the level of the plasma membrane (Dunphy et al., 1996; van't Hof & Resh, 1997).

We have recently shown (Schroeder et al., 1996) that cysteinyl-containing lipidated peptides can be S-acylated by mammalian cells, and that the intracellular loci of such S-acylation reactions can be visualized by fluorescence microscopy. We show here that in cultured mammalian fibroblasts the farnesylated C-terminal heptapeptide of mature N-*ras* shows a pattern of S-acylation/deacylation and a subcellular distribution strongly resembling those reported for intact N-*ras*. Moreover, experiments using reduced temperatures or the inhibitor brefeldin A to suppress vesicular membrane transport indicate that the primary locus of S-acylation of this peptide is the plasma membrane.

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[1] The terms 'S-acylation' and 'palmitoylation' are used equivalently in this paper, in accord with current practice in the literature, even though the long-chain *S*-acyl groups attached to cellular proteins are not in all cases exclusively palmitoyl residues.

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Taken together, the results of our biophysical and biochemical studies suggest that the mechanism of localization of mature N-*ras* to the plasma membrane may be similar to that postulated for other palmitoylated signaling proteins associated with this membrane, such as various nonreceptor tyrosine kinases and heterotrimeric G-protein α-subunits.

MATERIALS AND METHODS

Materials. Protected amino acids and coupling reagents were obtained from Novabiochem (La Jolla, CA) and Aldrich (Steinheim, Germany). Di-*trans*-farnesol and *trans*-geraniol (Sigma Chemical Co., St. Louis, MO) were converted to the bromides as described previously (Corey et al., 1972). Cell culture media were obtained from GIBCO RL (Burlington, Ontario). BSA[2] (fraction V, Sigma) was fatty acid-depleted by the method of Chen (1967).

The peptides Bimta-MGLPC(farn)-OMe and -BimGC-(palmitoyl)MGLPC(farn)-OMe were synthesized by solution coupling of the previously described peptide MGLPC(farn)-OMe (Stöber et al., 1997) with *S*-bimanylmercaptoacetic acid and Bimta-GC-palmitoyl-OH, respectively, using carbodiimide methods. The building block Bimta-GC(palmitoyl)-OH was synthesized starting from bis-Boc-protected bis-(glycylcystine) bisallyl ester by cleavage of the disulfide bond with dithiothreitol and S-acylation with palmitoyl chloride. After acid-mediated removal of the Boc group, *S*-bimanylmercaptoacetic acid was coupled to the free amino group, and the C-terminal allyl ester was cleaved by Pd(0)-mediated allyl transfer to morpholine as accepting nucleophile. Bimta-GCMGLPC(farn)-OMe was synthesized by solution coupling of FmocGC(StBu)-OH and MGLPC(farn)-OMe followed by deprotection of the N-terminal amino group and coupling to *S*-bimanylmercaptoacetic acid. The prenylated, StBu-protected peptide was finally S-deprotected with 1,4-butanedithiol as described previously (Shahinian & Silvius, 1995).

The peptide NBD-GCMGLPC(farn)-OMe was synthesized by benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate-mediated coupling of NBD-GC(StBu)MG-OH (synthesized by Fmoc/solid-phase chemistry and labeled with NBD-fluoride after cleavage and workup) and LPC(farn)-OMe [synthesized in solution starting from Cys(StBu)-OMe, then S-deprotected and prenylated with di-*trans*-farnesyl bromide in the presence of $KF{\cdot}2H_2O$, using methods described previously (Xue et al., 1991; Silvius & l'Heureux, 1994)]. The prenylated heptapeptide product was finally S-deprotected using 1,4-butanedithiol as noted above. Other S-alkylated heptapeptide analogues were synthesized in the same manner using the appropriate precursor peptides LPC-(alkyl)-OMe. Portions of the fluorescent-labeled heptapeptides were S-acylated with palmitoyl chloride as described previously (Quesnel & Silvius, 1994). NBD-GSMGLPC-(farn)-OMe and its geranylated analogue were synthesized by the same strategy as the corresponding cysteinyl peptides.

Methods. Large unilamellar lipid vesicles (LUV) were prepared by drying lipid mixtures under vacuum from chloroform/methanol stock solutions, resuspending in 150 mM NaCl, 5 mM Tes, 0.1 mM EDTA, pH 7.2 (except where otherwise indicated), and freeze-thawing 3 times before extruding through 0.1 μm pore size polycarbonate filters (MacDonald et al., 1991). Partitioning of bimane-labeled lipidated peptides between the aqueous phase and lipid vesicles was monitored by measuring the fluorescence of a fixed quantity of lipidated peptide in the presence of varying concentrations of LUV (egg PC/POPE, 90:10 mol/mol) as described previously (Silvius & l'Heureux, 1994), with the modification that the effective lipid concentration was equated to the total lipid concentration based on the finding that the peptides exhibit rapid transbilayer diffusion.

The kinetics of transfer of lipidated peptides between LUV were monitored by fluorescence measurements as described previously (Shahinian & Silvius, 1995), using a pH of 6.0 to ensure minimal hydrolysis ($<0.1\%$/h) of S-acylated peptides. Dithionite reduction of NBD-labeled lipidated peptides was monitored as the time-dependent fluorescence decrease when sodium dithionite was added to lipid vesicles incorporating 0.5 mol % lipidated peptide (McIntyre & Sleight, 1991). Very similar time courses of probe reduction were observed at dithionite concentrations from 2 to 30 mM, demonstrating that the results did not reflect permeation of dithionite into the vesicles (Langner & Hui, 1993).

S-^{3}H-Palmitoylation of prenylated peptides by cultured CV-1 fibroblasts was assayed as described previously (Schroeder et al., 1996) with minor modifications. Briefly, lipidated peptides (10 mM in DMF) were injected into 5 mM suspensions of sonicated POPC vesicles (9 mol of lipid/mol of lipopeptide) in serum-free medium (SFM: Dulbecco's minimum essential medium supplemented with 10 mM glutamine, 5 mM pyruvate, and 50 μg/mL gentamycin) plus 5 mM DTT, and the mixtures were incubated in the dark for 15 min at 37 °C under argon. Cell monolayers grown in 12-well dishes to 80−90% confluency were washed 3 times with SFM, and then incubated in SFM with the peptide-loaded vesicles (25 or 50 μM peptide, as indicated) and [^{3}H]-palmitic acid (160 μCi/mL, 3.7 μM). The final concentrations of DTT and of DMF in the cell−incubation mixtures were 0.5 mM and $\leq 0.5\%$ (v/v), respectively. After incubation, the cells were washed 3 times each in the cold with SFM containing 3 mg/mL BSA, SFM alone, and 150 mM NaCl, 20 mM HEPES, pH 7.0, and then suspended by scraping at 0 °C into 250 mM sucrose, 20 mM phosphate, 5 mM iodoacetamide, 1 mM EGTA, 1 mM PMSF, and 10 μg/mL each aprotinin, soybean trypsin inhibitor, and leupeptin, pH 7.4. The cell suspension was pelleted (1000*g*, 5 min), resuspended in the latter buffer, and finally lipid-extracted in an aqueous/methanol/methylene chloride two-phase system as described previously (Schroeder et al., 1996). The methylene chloride extract was concentrated under nitrogen, mixed with 2 nmol of S-acylated peptide standard, and separated by two-dimensional TLC on silica gel G plates (Whatman PE Sil G, Fisher Scientific, St. Laurent, Canada), developing with 90:10:0.2 (v/v/v) methylene chloride/methanol/acetic acid in the first dimension and ethyl acetate/acetic acid (199:1) in the second. The S-^{3}H-acylated peptide spot was visualized by fluorescence, scraped, and quantitated

[2] Abbreviations: acyl-CoA, acyl-coenzyme A ester; BSA, bovine serum albumin; N-oleoyl-DABS-PE, *N*-oleoyl-1-palmitoyl-2-[12-[[[[4[(4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]-stearoyl]phosphatidylethanolamine; DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid trisodium salt; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid sodium salt; LUV, large unilamellar vesicles; NBD-, 7-nitro-2,1,3-benzoxadiazolyl-; egg PC, egg yolk phosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; Rho-PE, *N*-(lissamine rhodamine sulfonyl)phosphatidylethanolamine; SFM, serum-free medium; TLC, thin-layer chromatography. Peptides are designated using the conventional one-letter amino acid code plus these additional abbreviations: Bimta-, *S*-bimanylmercaptoacetyl-; Boc-, *tert*-butoxycarbonyl-; -edNBD, (2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)ethyl)amino-; (farn), *S*-farnesyl; (StBu), *S*-*tert*-butylmercapto-.

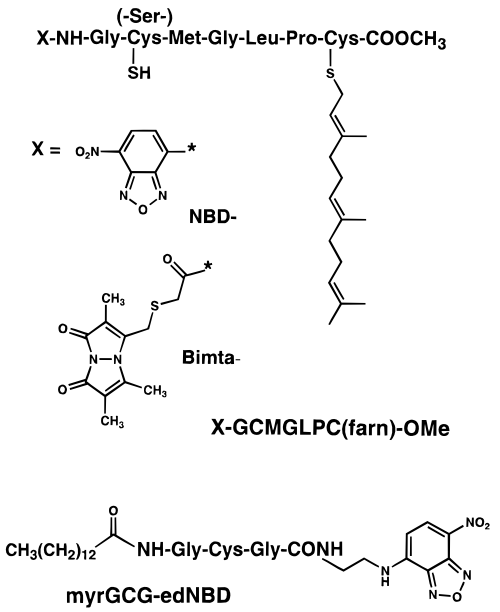

FIGURE 1: Structures of the prenylated peptides examined in this study. Other S-alkylated analogues of X-GCMGLPC(farn)-OMe were generated by replacing the indicated farnesyl group as discussed in the text.

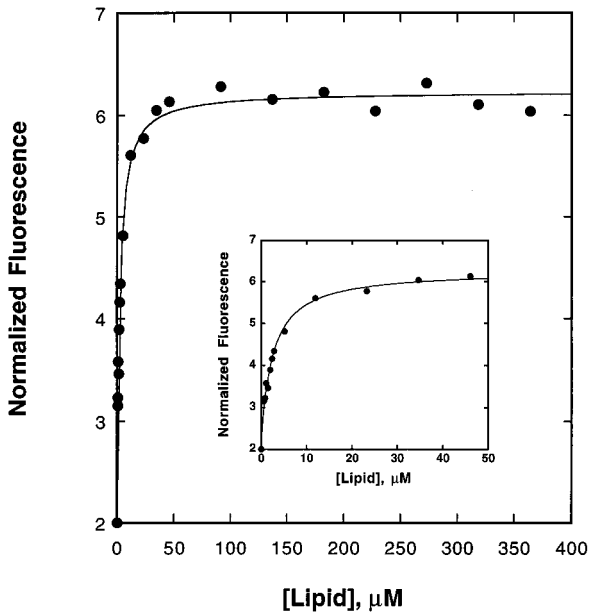

FIGURE 2: Fluorescence monitoring of the partitioning of Bimta-GCMGLPC(farn)-OMe into LUV (diameter ca. 100 nm) composed of egg PC/POPE (90:10 molar proportions). The fluorescence of a constant amount of lipidated peptide was determined in the presence of variable amounts of lipid vesicles, and the resulting titration curve was analyzed as described in the text. (Inset: lower range of curve.)

by scintillation counting in Cytoscint (ICN Canada, St. Laurent, Québec) after a 24-h incubation. Sample protein concentrations were measured as described previously (Schroeder et al., 1996).

For fluorescence microscopy, CV-1 cells were incubated in Hanks' buffered saline containing 0.5 mM DTT and 4 mM sonicated POPC vesicles incorporating 100 μM NBD-labeled lipidated peptide. After incubation for the indicated times, the cells were washed twice briefly and once for 1 h at 4 °C in Hanks' buffered saline containing BSA (5% w/v), and then immediately observed using a Zeiss EM 35 inverted epifluorescence microscope equipped with an MC100 camera. Control experiments carried out as described previously (Schroeder et al., 1996) confirmed that this albumin washing effectively removed the unacylated form of the peptide.

RESULTS

Biophysical Properties of the N-ras C-Terminal Peptide. In Figure 1 are shown the structures of the fluorescent-labeled prenylated peptides examined in this study. The sequence -GCMGLPC(farn)-OMe represents the C-terminus of mature N-*ras*, where the underlined cysteine residue corresponds to the physiologically acylated Cys-181 in the mature protein (Hancock et al., 1991). Bimane-labeled peptides were used for the biophysical experiments described in this section because previous results (Skerjanc et al., 1987; Silvius & l'Heureux, 1994) have shown that the presence of a bimane group does not significantly alter the affinity of association of labeled peptides with lipid bilayers.

The partitioning of bimane-labeled prenylated peptides into lipid bilayers was assessed by monitoring the enhancement of fluorescence of these species as a function of the concentration of added lipid vesicles, which can be analyzed to yield a (dimensionless) mole fraction-based partition coefficient K_p (Silvius & l'Heureux, 1994). A representative determination for the N-*ras* C-terminal peptide Bimta-GCMGLPC(farn)-OMe is shown in Figure 2. From such data, we estimate that for this species $K_p = (4.4 \pm 0.4) \times 10^6$ and that K_d^{eff}, the lipid concentration at which 50% of the lipidated peptide is vesicle-bound, is 1.36 ± 0.57 μM. This K_d^{eff} value is significantly lower than those determined previously for farnesylated/O-methylated peptides with more hydrophilic side chains (Silvius & l'Heureux, 1994; Ghomashchi et al., 1995). The shorter peptide Bimta-MGLPC-(farn)-OMe bound to lipid vesicles with a similar affinity (not shown). In mammalian cells, where the effective concentration of membrane lipids exposed to the cytoplasm is of the order of several millimolar (Griffiths et al., 1989), the C-terminus of mature but unpalmitoylated N-*ras* should thus be sufficiently hydrophobic to promote efficient association of the protein with membrane lipid bilayers.

The kinetics of spontaneous (diffusional) interbilayer transfer of the mature N-*ras* C-terminal heptapeptide, in both its S-palmitoylated and its unacylated forms, were assessed using a fluorescence assay. To donor POPC LUV, incorporating 1 mol % of the nonexchangeable fluorescence quencher *N*-oleoyl-DABS-PE and 0.4 mol % of bimane-labeled lipidated peptide, was added a 9-fold excess of sonicated POPC 'acceptor' vesicles, and the time course of subsequent transfer of the lipidated peptide from donor to acceptor vesicles was monitored by fluorescence dequenching as described previously (Nichols & Pagano, 1982; Silvius & Zuckermann, 1993; Shahinian & Silvius, 1995). The complete time course of transfer represents two component processes: transfer to acceptor vesicles of heptapeptide molecules initially present at the *outer* surfaces of donor vesicles, and transbilayer flip-flop, followed by transfer to acceptor vesicles, of heptapeptide molecules initially present at the *inner* surfaces of donor vesicles [discussed for related systems by Homan and Pownall (1988) and Wimley and Thompson (1990)]. As shown in Figure 3A, the overall time course of donor-to-acceptor transfer of Bimta-GCMGLPC-(farn)-OMe was rapid, proceeding to equilibrium within a few minutes. We can thus conclude that the half-times for both intervesicle transfer and transbilayer diffusion of the prenylated heptapeptide are of the order of seconds at 37 °C.

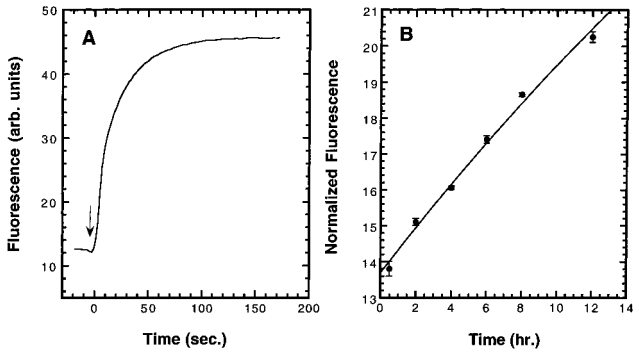

FIGURE 3: (Panel A) Time course of intervesicle transfer of Bimta-GCMGLPC(farn)-OMe at 37 °C. To LUV (POPC, 30 μM) containing the labeled lipidated peptide (1 mol %) and the nonexchangeable fluorescence quencher DABS-PC (2 mol%) was added a 10-fold excess of unlabeled vesicles at time zero. Transfer of the lipidated peptide from donor to acceptor vesicles was monitored by the subsequent time-dependent enhancement of fluorescence as described in the text. (Panel B) Time course of exchange of S-palmitoylated Bimta-GCMGLPC(farn)-OMe between POPC LUV. The maximum (infinite-time) fluorescence value in the experiment shown was determined to be 46.7 from parallel control experiments as described in Shahinian and Silvius (1995).

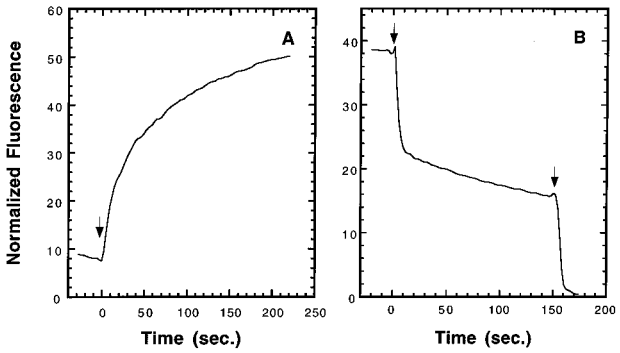

FIGURE 4: (Panel A) Time course of intervesicle transfer of NBD-GCMGLPC(farn)-OMe at 37 °C. To POPC LUV (30 μM) containing the labeled lipidated peptide (1 mol %) and the nonexchangeable fluorescence quencher Rho-PE (2 mol%) was added a 10-fold excess of unlabeled vesicles at time zero. Other experimental details were as described for Figure 3B. (Panel B) Time course course of reduction of NBD-GCMGLPC(farn)-OMe (1 mol % in 30 μM POPC LUV) by sodium dithionite (10 mM) at 37 °C; 0.5% Triton X-100 was added at $t = 150$ s. Other experimental details were as described under Materials and Methods.

In contrast to the behavior observed for Bimta-GCMGLPC(farn)-OMe, spontaneous interbilayer diffusion of the S-palmitoylated derivative was extremely slow, as illustrated in Figure 3B. The first-order rate constant for spontaneous interbilayer transfer of the palmitoylated species at 37 °C was determined to be $(3.7 \pm 0.4) \times 10^{-2}$ h^{-1}, corresponding to a half-time of ca. 19 h. This very slow rate of spontaneous interbilayer diffusion is similar to that observed previously for a peptide analogous to the palmitoylated/farnesylated C-terminus of mature H-*ras* (Shahinian & Silvius, 1995). Based on the present results and on previous comparisons of the kinetics of interbilayer transfer of lipid-conjugated small molecules *vs* macromolecules (Silvius & Zuckermann, 1993), we can estimate that the half-time for spontaneous intermembrane transfer of mature, S-acylated N-*ras* would be on the order of several hours.

Inter- and Transbilayer Diffusion of the NBD-Labeled N-ras C-Terminal Peptide. To interpret the studies described later of the S-acylation and localization of the NBD-labeled C-terminal peptide of N-*ras* in mammalian cells, it was important to verify that this species can rapidly diffuse between and across different membrane bilayers in its unacylated form but *cannot* readily diffuse between different membranes when S-acylated. These points were addressed using two approaches. In the first, as described above for the bimane-labeled farnesyl-peptide, the NBD-labeled species was incorporated into POPC donor LUV together with the nonexchangeable fluorescence quencher Rho-PE, and the kinetics of intervesicle peptide transfer were monitored by fluorescence enhancement after adding a 9-fold excess of 'acceptor' vesicles composed of POPC alone. As shown in Figure 4A, the time course of intervesicle peptide exchange was biphasic and could be well-described as a sum of two exponentials (fitted curve not shown). For NBD-GCMGLPC(farn)-OMe at 37 °C, a half-time of 21 ± 6 s (mean of three determinations) was estimated for the rapid phase, representing transfer of lipidated peptide molecules between the vesicles' outer surfaces, while a half-time of 1.8 ± 0.6 min was estimated for the slow phase, which is rate-limited primarily by the translocation of peptide molecules from the inner to the outer leaflet of the donor vesicles. At 15 °C, the estimated half-times for inter- and transbilayer diffusion increased to 3.2 ± 0.7 and 13.9 ± 1.1 min, respectively. Transfer and flip-flop rates of comparable magnitude were determined for the analogous serinyl-peptide NBD-GSMGLPC(farn)-OMe (not shown). Much faster rates of interbilayer diffusion were measured for the geranylated species NBD-GCMGLPC(geranyl)-OMe (half-times <1 s at 37 °C and 8 ± 3 s at 15 °C), in accord with the lower hydrophobicity of the geranyl group compared to the farnesyl group.

To corroborate the conclusion that transbilayer diffusion of NBD-GCMGLPC(farn)-OMe is rapid, we examined the kinetics of dithionite reduction of the peptide incorporated in POPC LUV (McIntyre & Sleight, 1991; Balch et al., 1994). As illustrated in Figure 4B, extravesicular dithionite rapidly reduces probe molecules initially present at the vesicles' outer surfaces, quenching their fluorescence. The residual fluorescence then decreases more slowly as peptide molecules diffuse from the vesicles' inner leaflets to their outer surfaces and there undergo rapid reduction. The half-time estimated from this slower phase of dithionite reduction for the transbilayer diffusion of NBD-GCMGLPC(farn)-OMe is 1.7 ± 0.6 min (mean of five determinations) at 37 °C, in good agreement with the value derived from the above peptide-transfer measurements.

The acylated/farnesylated peptide NBD-GC(palmitoyl)-MGLPC(farn)-OMe was found to transfer between lipid bilayers with extremely slow kinetics (estimated half-time 155 ± 50 h). The slower interbilayer diffusion of the NBD-labeled vis-à-vis the bimane-labeled species may reflect the fact that the NBD group itself modestly enhances the association of NBD-labeled molecules with lipid bilayers (Nichols, 1985). Taken together, the above results demonstrate that the NBD-labeled peptides discussed in the next section diffuse rapidly between and across lipid bilayers when prenylated only but show extremely slow rates of interbilayer diffusion when palmitoylated as well.

S-Acylation and Membrane Targeting of the N-ras C-Terminal Heptapeptide and Analogues in Cultured Mammalian Fibroblasts. We have previously shown that a variety of N-myristoylated cysteinyl-containing peptides are converted to S-acyl derivatives in intact mammalian cells and accumulate in this form within specific cellular membranes

Table 1: S-[^{3}H]Acylation of Lipopeptides by CV-1 Cells at 37 °C

lipopeptide	S-[^{3}H]acylated peptide formed (cpm/μg of protein)[a]
NBD-GCMGLPC(farnesyl)-OMe	8.8 ± 1.1
NBD-GSMGLPC(farnesyl)-OMe	0.7 ± 0.1
NBD-GCMGLPC(geranyl)-OMe	11.2 ± 1.4
NBD-GSMGLPC(geranyl)-OMe	0.6 ± 0.1
myristoyl-GCG-edNBD	208.5 ± 7.3
none	0.6 ± 0.1

[a] Monolayers of CV-1 cells grown in 12-well multiwell dishes were incubated with [^{3}H]palmitate (40 μCi/mL) and the indicated lipopeptides in sonicated POPC vesicles (50 μM in 450 μM lipid). After incubation for 2 h at 37 °C, the monolayers were washed, and formation of S-[^{3}H]acylated peptide was determined as described under Materials and Methods. The blank value shown as 'none' was determined as the radioactivity migrating with the palmitoylated NBD-GCMGLPC(farn)-OMe standard in control incubations without peptide; similar blank values were measured at the positions of the other palmitoylated standards after similar incubations.

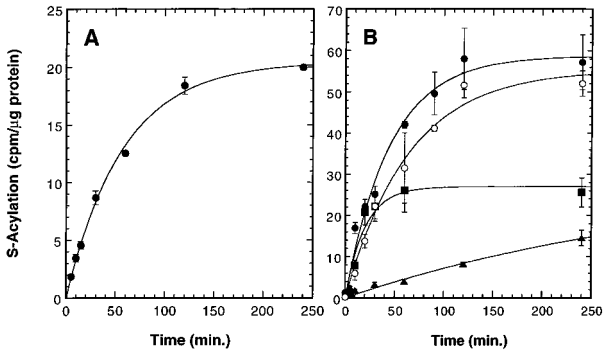

FIGURE 5: (Panel A) Time course of S-acylation of NBD-GCMGLPC(farn)-OMe by CV-1 cells at 37 °C. To washed cell monolayers were added [^{3}H]palmitic acid (160 μCi/mL) and lipidated peptide (50 μM in 450 μM sonicated POPC vesicles). After the indicated times of incubation, the cells were washed, total cell-associated lipidated peptides (and lipids) were extracted and separated by TLC, and S-^{3}H-acylated peptide was quantitated by scintillation counting as described under Materials and Methods. Values shown represent the mean of triplicate values determined in one experiment; four separate experiments of this type were carried out to provide the results discussed in the text. Panel (B) Time courses of the S-acylation of S-alkylated heptapeptides NBD-GCMGLPC(alkyl)-OMe, where (alkyl) = (○) farnesyl, (●) *n*-octyl, (■) *n*-undecyl, or (▲) methyl. Incubations were carried out as for panel A but adding 320 μCi/mL [^{3}H]palmitate to the incubation mixtures. Other experimental details for panels A and B were as described under Materials and Methods.

(Schroeder et al., 1996). We therefore investigated the intracellular metabolism and subcellular localization of the mature N-*ras* C-terminal heptapeptide in CV-1 (simian kidney fibroblastic) cells in culture.

When CV-1 cells are incubated with [^{3}H]palmitate and NBD-GCMGLPC(farn)-OMe, using sonicated lipid vesicles as a carrier, production of the S-^{3}H-palmitoylated peptide can be readily detected by thin-layer chromatography and autoradiography as described under Materials and Methods. By contrast, no significant acylation of the analogous serinyl-lipopeptide NBD-GSMGLPC(farn)-OMe was detected under the same conditions (Table 1). As shown in Figure 5A, upon simultaneous addition of the cysteinyl-peptide and [^{3}H]-palmitate to the cells at 37 °C, formation of S-^{3}H-palmitoylated peptide begins at a maximal rate at the earliest time points sampled and plateaus at longer times. Significant S-acylation of the peptide was also observed at 15 °C and even at 4 °C, at rates roughly 2- and 7-fold slower than at 37 °C (not shown).

The time course shown in Figure 5A can be well-described by the following equation, derived assuming that the intracellular concentration of the unacylated peptide substrate rapidly reaches its steady-state level:

$$([^3\text{H}]\text{acylpeptide}) = A[1 - \exp(-k_{\text{turn}}t)]$$

where A is the steady-state level of acylpeptide formed and k_{turn} is the rate constant for turnover of the S-^{3}H-acylated peptide. From three independent experiments, we estimated the value of k_{turn} as 1.06 ± 0.06 h^{-1}, corresponding to a half-time of 39 min for turnover of the S-acylated form of the peptide. A similar rate of turnover was estimated by pulse−chase experiments; when cells were incubated for 2 h with [^{3}H]palmitate (160 μCi/mL, 3.7 μM) and NBD-GCMGLPC-(farn)-OMe as above, and then transfered to SFM containing only unlabeled palmitate (370 μM, in 3.7 mM sonicated POPC vesicles), the level of [^{3}H]acylated peptide decreased by 55% in 1 h. By contrast, <10% loss of ^{3}H-labeled peptide was observed in 4 h when the chase was carried out in the absence of unlabeled palmitate, indicating that the decrease in [^{3}H]acylpeptide was due to turnover of the *S*-acyl group and not to degradation of the peptide. A slightly longer half-time (ca. 50 min) was estimated for turnover of the *S*-acyl group at 15 °C (not shown).

The rate of *S*-acyl group turnover observed for the C-terminal heptapeptide of mature N-*ras* is considerably faster than that observed for previously studied cysteinyl-lipopeptides such as myristoyl-GCG-edNBD (see structure in Figure 1), whose S-acylated form exhibits a turnover half-time of several hours [Schroeder et al., 1996; and this study (not shown)]. Partly reflecting this fact, the steady-state level of *S*-[^{3}H]acylpeptide accumulated in cells incubated with NBD-GCMGLPC(farn)-OMe for 2 h (Table 1) was some 20-fold lower than that in cells incubated with myrGCG-edNBD under the same conditions. However, the estimated initial rate of S-acylation of NBD-GCMGLPC(farn)-OMe is only about 3.5-fold lower than that measured for S-acylation of myrGCG-edNBD in parallel experiments [0.87 cpm min^{-1}/(μg of protein)$^{-1}$ *vs* 3.12 cpm min^{-1}/(μg of protein)$^{-1}$, respectively, under the conditions described for Figure 5B]. The C-terminal peptide of N-*ras* is thus a relatively efficient substrate for S-acylation but also shows a relatively high rate of *S*-acyl turnover. Interestingly, the geranylated heptapeptide NBD-GCMGLPC(geranyl)-OMe was S-acylated to a slightly greater extent than the farnesylated species (Table 1), while the analogous serine-containing geranyl-peptide was again not detectably acylated (Table 1).

In order to explore further the importance of the prenyl group for S-acylation of the mature N-*ras* C-terminal heptapeptide, we examined the S-acylation of a series of heptapeptide analogues in which the farnesyl residue was replaced by a saturated *n*-alkyl group. As shown in Figure 5B, heptapeptide analogues bearing *S*-undecyl-, *S*-octyl-, or even *S*-methyl substituents are all substrates for S-acylation. The analogue bearing an *S*-undecyl group, which is similar in hydrophobicity to a farnesyl group (Silvius & l'Heureux, 1994), is S-acylated at an initial rate similar to that observed for the farnesylated peptide but yields somewhat less S-acylated product at steady-state. The *S*-octyl-substituted heptapeptide shows both an initial rate and a steady-state level of *S*-acylpeptide formation that are very similar to those observed for the farnesylated species. The S-methylated heptapeptide, which showed a weak but measurable affinity

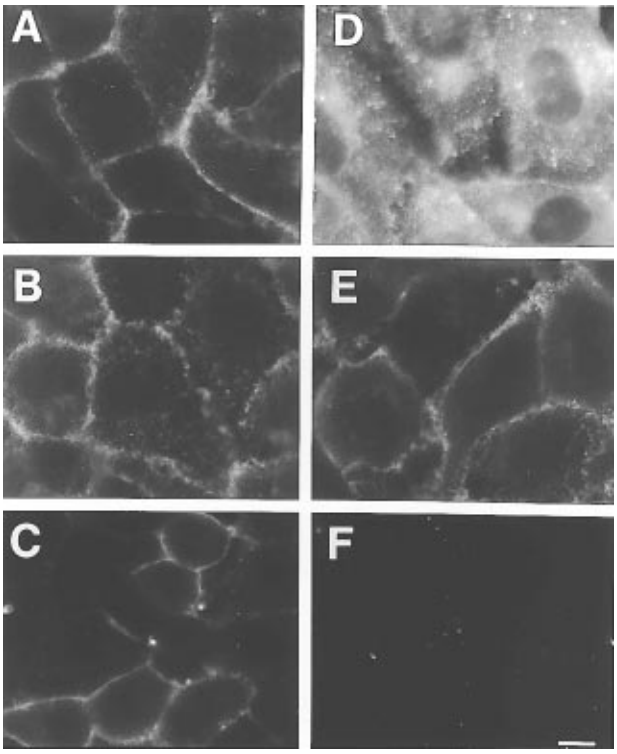

FIGURE 6: Fluorescence micrographs of CV-1 cells incubated with NBD-labeled lipidated peptides (100 μM in 2 mM sonicated POPC vesicles) and then washed extensively with albumin in the cold as described under Materials and Methods. (Panels A and B) Cells incubated with NBD-GCMGLPC(farn)-OMe for 12 h at 15 °C (panel A) or 2 h at 37 °C (panel B). (Panel C) Cells incubated with NBD-GCMGLPC(farn)-OMe for 2 h at 37 °C in the presence of brefeldin A (5 μg/mL, added 1 h before and maintained during the incubation with peptide). (Panel D) Cells incubated at 37 °C as in (A) but then visualized *without* albumin washing to remove unacylated peptide. (Panel E) Cells incubated with NBD-GCMGLPC(geranyl)-OMe for 7 h at 15 °C. (Panel F) Cells incubated with NBD-GSMGLPC(geranyl)-OMe for 7 h at 15 °C. Space bar = 10 μM.

for lipid bilayers in control experiments (data not shown), was also S-acylated by CV-1 cells, but with much slower kinetics than more hydrophobic heptapeptide derivatives (Figure 5B). Taken together, these data indicate that the S-acylation reaction, and the turnover of the *S*-acyl group, within CV-1 cells exhibit no marked selectivity for the farnesylated peptide over analogues bearing other *S*-alkyl groups, so long as the latter associate efficiently with lipid bilayers.

The subcellular locus of S-acylation of NBD-GCMGLPC-(farn)-OMe and of its geranylated analogue was examined using a previously described fluorescence−microscopic approach (Schroeder et al., 1996). In their unacylated form, these peptides are efficiently extracted from cultured CV-1 cells by repeated washing with serum albumin at low temperature (results not shown). By contrast, in their S-acylated form, the peptides are highly refractory to albumin extraction and (as discussed above) to spontaneous intermembrane transfer. During incubation of cells with the farnesylated peptide, the S-acylated form of the peptide will thus accumulate specifically in the membrane compartment(s) in which it is generated (and, potentially, in compartments to which it may subsequently be transfered by vesicular membrane traffic). These compartments can then be visualized selectively by fluorescence microscopy after albumin extraction of unacylated lipopeptide.

In Figure 6A and Figure 6B are shown fluorescence micrographs of CV-1 cells incubated with NBD-GCMGLPC-(farn)-OMe at 15 °C (12 h) or 37 °C (2 h), respectively, and then washed with albumin as discussed above. To maximize the extent of *S*-acylpeptide formation at 15 °C, cells were incubated with the prenylated peptide at this temperature for up to 12 h. However, similar (but fainter) patterns of cell-associated fluorescence were observed using cells incubated for shorter times (2−4 h) at 15 °C. After incubation at either temperature, the cells reveal a preferential accumulation of S-acylated lipopeptide at the plasma membrane. Treatment of the cells with brefeldin A (5 μg/mL), which inhibits anterograde transport of materials within the Golgi apparatus and, in some cases, from the trans-Golgi network to the plasma membrane as well (Klausner et al., 1992; Miller et al., 1992; Donaldson & Klausner, 1994), did not perturb the preferential plasma membrane localization of the S-acylated species (Figure 6C) and caused only a slight (<15%) reduction in the steady-state level of peptide acylation (not shown). The observation that the S-acylated peptide is localized to the plasma membrane when generated in the presence of brefeldin or during incubation at 15 °C, where most processes of vesicular transfer of materials between membranes are suppressed (Matlin & Simons, 1983; Saraste & Kuismanen, 1984; Kaplan & Simoni, 1985; Pagano et al., 1991; Shiao & Vance, 1993; Connolly et al., 1994), indicates that the plasma membrane is itself a major locus of S-acylation of the N-*ras* C-terminal peptide.

In contrast to the results just described, CV-1 cells incubated with the N-*ras* C-terminal peptide and visualized *without* subsequent albumin washing reveal significant fluorescence labeling of intracellular membranes as well as the plasma membrane (Figure 6D). TLC analysis (not shown) of fluorescent materials extracted from cells after incubation with the farnesylated (or geranylated) peptide revealed only the unacylated peptide, its S-acylated derivative, and trace amounts of disulfide-linked peptide dimer, of which only the unacylated peptide was removed by albumin washing. The microscopic result illustrated in Figure 6D thus indicates that under our experimental conditions the exogenously added farnesyl-peptide, which presumably first encounters the plasma membrane during cellular uptake, in its unacylated form can gain access to intracellular membranes as well. This finding, combined with our observation that the S-acylated peptide preferentially accumulates in the plasma membrane at 15 °C (where as already noted most membrane-trafficking pathways are suppressed), suggests that the activity responsible for S-acylation of the N-*ras* C-terminal peptide is highly enriched in the plasma membrane in comparison to other, intracellular membrane compartments.

The geranylated analogue of the N-*ras* C-terminal peptide, NBD-GCMGLPC(geranyl)-OMe, was also preferentially localized to the plasma membrane in its S-acylated form in CV-1 cells (Figure 6E). In contrast, cells incubated with the analogous serinyl-lipopeptide NBD-GSMGLPC(geranyl)-OMe and washed with albumin showed little residual fluorescence (Figure 6F). Cells incubated with these geranyl-peptides and then visualized without albumin washing showed intracellular as well as plasma membrane fluorescence (not shown), indicating that like their farnesylated counterparts the geranylated peptides have access to intracellular membranes in their unacylated form. These results are consistent with our above biophysical observations that

the prenylated but unacylated peptides can redistribute rapidly between different membranes.

DISCUSSION

The biophysical properties of the C-terminal heptapeptide of mature (farnesylated/methylated) N-*ras* suggest that the mature protein can be localized to specific membranes by a kinetic trapping mechanism (Shahinian & Silvius, 1995), as outlined below. The farnesylated but unpalmitoylated C-terminal sequence of mature N-*ras* exhibits efficient but rapidly reversible association with lipid bilayers. By contrast, additional S-acylation of this sequence renders the membrane anchorage effectively irreversible (in the kinetic sense) for the lifetime of the S-acylated species. These biophysical properties are discussed later in this section in the context of the plasma membrane targeting of mature N-*ras*.

The farnesylated C-terminal heptapeptide of mature N-*ras* is readily palmitoylated by cultured mammalian cells, consistent with previous findings that the extreme C-terminal segment of this protein can direct S-acylation of a heterologous protein (Lu & Hoffman, 1995). Several points argue that the mechanism of this S-acylation is enzymatic. First, as we have discussed previously for the S-acylation of other peptides by CV-1 cells, the rapid initial kinetics of S-^{3}H-acylpeptide formation upon simultaneous addition of peptide substrate and [^{3}H]palmitate are inconsistent with a nonspecific abstraction of *S*-acyl groups from cellular proteins, which incorporate [^{3}H]palmitate only over periods of hours (Schroeder et al., 1996). Second, we have recently shown that the rates of nonenzymic S-acylation of cysteinyl-containing lipidated peptides are very slow in the presence of physiological concentrations of acyl-CoAs, membrane lipids, and acyl-CoA binding proteins (Leventis et al., 1997; Leventis and Silvius, unpublished results), suggesting that the S-acylation by CV-1 cells of the peptides examined here is very unlikely to result from a nonenzymic reaction with long-chain acyl-CoAs. The specific localization of the S-acylation reaction to the plasma membrane similarly appears to be inconsistent with a nonenzymic reaction of the peptide with either cytoplasmic or membrane-bound long-chain acyl-CoAs.

Our findings using various *S*-alkyl analogues of the N-*ras* C-terminal heptapeptide indicate that the farnesyl group *per se* is not a crucial determinant for S-acylation of this peptide at the plasma membrane. The farnesyl residue may instead promote S-acylation simply by favoring membrane association of the N-*ras* C-terminus, thereby exposing this region of the protein to a membrane-bound *S*-acyltransferase activity. A similarly generalized role has been proposed for the N-terminal myristoyl residue of certain heterotrimeric G-protein α-subunits in promoting S-acylation of these proteins on nearby cysteine residues (Degtyarev et al., 1993, 1994; Linder et al., 1993; Wilson & Bourne, 1995).

The rate of turnover of the *S*-[^{3}H]palmitoyl group incorporated into NBD-GCMGLPC(farn)-OMe in CV-1 cells ($t_{1/2}$ = 35−40 min at 37 °C) is almost as rapid as that reported for full-length N-*ras* [$t_{1/2}$ = ca. 20 min (Magee et al., 1987)], suggesting that the rapid *S*-acyl turnover observed for N-*ras* is largely determined by properties intrinsic to the extreme C-terminus of the protein. This conclusion is consistent with the results of Lu and Hoffman (1995), who found that an N-*ras*/protein A chimera containing the C-terminal 12 amino acids of N-*ras* also exhibited rapid *S*-acyl turnover.

The observation that the palmitoylated C-terminal heptapeptide of mature N-*ras* accumulates predominantly in the plasma membrane of CV-1 cells agrees with the previous finding by immunofluorescence that N-*ras* is preferentially localized to this membrane (Grand et al., 1987). Previous studies have suggested that the plasma membrane targeting information for the related protein H-*ras* also resides in the extreme C-terminal segment of the protein, which includes two sites of S-acylation (Hancock et al., 1991). Gutierrez and Magee (1991) have reported that an activity capable of S-acylating mature N-*ras* in vitro is associated not with the plasma membrane fraction but rather with membranes tentatively identified as a Golgi subfraction. This result could suggest, as noted by these latter authors, that upon deacylation N-*ras* is reacylated at the level of the Golgi apparatus and then recycled to the plasma membrane by vesicular trafficking. However, our biochemical and fluorescence−microscopic results, particularly those obtained using reduced temperatures or brefeldin A to suppress intra-Golgi and/or trans-Golgi-to-plasma-membrane trafficking pathways (Matlin & Simons, 1983; Saraste & Kuismanen, 1984; Kaplan & Simoni, 1985; Pagano et al., 1991; Shiao & Vance, 1993; Connolly et al., 1994; Klausner et al., 1992; Miller et al., 1992; Donaldson & Klausner, 1994), indicate that the plasma membrane itself incorporates an S-acyltransferase activity that can efficiently acylate the C-terminal sequence of mature N-*ras*. Our failure to observe substantial accumulation of the S-acylated peptide in other identifiable cellular compartments (notably the Golgi complex), even under conditions where various intracellular trafficking pathways are suppressed, suggests moreover that the plasma membrane may in fact be the *principal* cellular locus of S-acylation in the deacylation/reacylation cycle of mature N-*ras*. In this regard, mature N-*ras* appears to resemble proteins such as p59^{fyn} and the α-subunits of various G-proteins, which are also apparently S-acylated at the level of the plasma membrane (Dunphy et al., 1996; van't Hof & Resh, 1997).

Our results also raise the possibility that S-acylation at the plasma membrane could function to retrieve to the plasma membrane mature N-*ras* molecules that in their deacylated form (free or possibly bound to cytoplasmic proteins) may escape to other cellular compartments during cyclical turnover of the *S*-acyl group. While the possibility that deacylated N-*ras* can dissociate from the plasma membrane has not been directly assessed, the mature but unpalmitoylated form of the related protein H-*ras* is readily released from cell membranes (Hancock et al., 1990; Willumsen et al., 1996), and a green fluorescent protein-tagged derivative of K-*ras*4B (which is not palmitoylated) exhibits a very rapid association/dissociation equilibrium with the plasma membrane (Yokoe & Meyer, 1996). The facile membrane dissociation observed for these related proteins suggests that in its deacylated form mature N-*ras* may also be able to dissociate to some extent from the plasma membrane. In this case, a preferential reacylation of the protein at the plasma membrane could still ensure the steady-state accumulation of the protein at this locus, even in the face of repeated turnover of the protein-linked *S*-acyl group.

As already noted, the plasma membrane-associated S-acyltransferase activity observed here is in principle sufficient to explain how the plasma membrane localization of mature N-*ras* is maintained, even if the deacylated protein may transiently dissociate from the plasma membrane. However, it cannot be excluded that N-*ras* may become S-acylated in

compartments other than the plasma membrane under particular circumstances, notably during the initial biosynthesis and maturation of the protein. N-*ras*, like other *ras* isoforms, is synthesized with a C-terminal -CAAX sequence that is successively modified by farnesylation, endoproteolysis, and carboxyl-methylation to yield the mature protein [for reviews, see Schafer and Rine (1992), Clark (1992), Newman and Magee (1993), Casey and Seabra (1996), and Zhang and Casey (1996)]. While the responsible farnesyltransferase is a cytosolic enzyme, the latter two reactions take place on membranes that cofractionate with markers for the endoplasmic reticulum and for the endoplasmic reticulum and plasma membrane, respectively (Stephenson & Clark, 1990; Jang et al., 1993; Pillinger et al., 1994; Li et al., 1996). The results of our present experimental system, in which the C-terminal peptide of N-*ras* is presented to cells in its already fully-processed form, do not exclude the possibility that during its normal course of maturation N-*ras* may be directed to an intracellular compartment in which it undergoes initial S-acylation before reaching the plasma membrane. Further study of the intracellular trafficking and S-acylation of newly biosynthesized N-*ras* should allow this possibility to be assessed.

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