Lipid-modified, Cysteinyl-containing Peptides of Diverse Structures Are Efficiently S-Acylated at the Plasma Membrane of Mammalian Cells

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Abstract. A variety of cysteine-containing, lipid-modified peptides are found to be S-acylated by cultured mammalian cells. The acylation reaction is highly specific for cysteinyl over serinyl residues and for lipid-modified peptides over hydrophilic peptides. The S-acylation process appears by various criteria to be enzymatic and resembles the S-acylation of plasma membrane–associated proteins in various characteristics, including inhibition by tunicamycin. The substrate range of the S-acylation reaction encompasses, but is not limited to, lipopeptides incorporating the motifs myristoylGC- and -CXC(farnesyl)-OCH_3, which are reversibly S-acylated in various intracellular proteins. Mass-spectrometric analysis indicates that palmitoyl residues constitute the predominant but not the only type of S-acyl group coupled to a lipopeptide carrying the myristoylGC- motif, with smaller amounts of S-stearoyl and S-oleoyl substituents also detectable. Fluorescence microscopy using NBD-labeled cysteinyl lipopeptides reveals that the products of lipopeptide S-acylation, which cannot diffuse between membranes, are in almost all cases localized preferentially to the plasma membrane. This preferential localization is found even at reduced temperatures where vesicular transport from the Golgi complex to the plasma membrane is suppressed, strongly suggesting that the plasma membrane itself is the preferred site of S-acylation of these species. Uniquely among the lipopeptides studied, species incorporating an unphysiological *N*-myristoylcysteinyl- motif also show substantial formation of S-acylated products in a second, intracellular compartment identified as the Golgi complex by its labeling with a fluorescent ceramide. Our results suggest that distinct S-acyltransferases exist in the Golgi complex and plasma membrane compartments and that S-acylation of motifs such as myristoylGC- occurs specifically at the plasma membrane, affording efficient targeting of cellular proteins bearing such motifs to this membrane compartment.

A VARIETY of integral membrane proteins and reversibly membrane-associated proteins in eukaryotic cells exhibits posttranslational acylation on one or more cysteine residues, a modification that for a number of such proteins appears to be dynamic (6, 39, 42, 45, 81, 82, 84) and, in some cases, is modulated by physiological or pharmacological stimuli (15, 31, 45, 46, 63, 86). Integral membrane proteins may be S-acylated either on cysteine residues near the cytoplasmic termini of transmembrane helixes (3, 14, 28, 30, 32, 68, 74) or on cytoplasmic cysteine residues more distant from a transmembrane helix (9, 18, 21, 88). Among the reversibly membrane-associated proteins that undergo S-acylation are found a number of *src*-homologous nonreceptor tyrosine kinases, heterotrimeric G protein α subunits and monomeric G proteins (for reviews see 10, 43, 44, 61, 65, 75). S-acylation has been shown to enhance the membrane association of a variety of proteins of the latter type and thereby to contribute to their physiological function (2, 11, 22, 25, 41, 51, 81, 83, 84, 86, 87, 90, 91).

To date, relatively little is known about the mechanism(s) and the subcellular loci of protein S-acylation. Based on the evidence of kinetic and cellular-fractionation studies, S-acylation of some viral and cellular integral membrane proteins has been suggested to occur in the Golgi complex, the endoplasmic reticulum, and/or an intermediate compartment between these two structures (for reviews see 68, 69). However, a variety of S-acylated proteins appears to be associated with the plasma membrane (9, 11, 16, 18, 22, 25, 35, 41, 46, 62, 77, 78, 86, 89), and the dynamic nature of this modification suggests that S-acylation may also occur either in the plasma membrane or in a membrane compartment that rapidly communicates with this membrane. Apparently enzymic activities mediating S-acylation of protein substrates have been identified in several intact or solubilized membrane preparations (1, 4, 19, 24, 70, 72) but to date have not been characterized at a molecular level. Certain integral membrane proteins (7,

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48, 66) and even simple (lipo)peptides (5, 27, 57) have been shown to exhibit spontaneous S-acylation in vitro in the presence of long chain acyl-CoAs, although it remains to be determined whether such autocatalytic reactions can mediate the S-acylation of proteins in intact cells.

A variety of eukaryotic intracellular proteins bearing cysteine residues near terminal *N*-myristoylglycyl- or S-prenylcysteinyl- residues are S-acylated in vivo (for review see 10, 43, 61, 65, 75), even when such motifs are introduced artificially into chimeric or mutated proteins (2, 4, 26, 77, 78). These findings suggested that simple lipopeptides containing such minimal motifs might serve as cell-permeant substrates to examine S-acylation in situ in eukaryotic cells. We here demonstrate that such lipopeptides, with structures resembling those of the lipidated terminal sequences of various intracellular proteins, are efficiently and specifically acylated on cysteine residues by cultured mammalian cells, by a process whose properties resemble in a variety of aspects those observed for the S-acylation of cellular proteins. Fluorescence microscopy reveals that most of these lipopeptides undergo S-acylation preferentially at the plasma membrane. However, a small subset of lipopeptides containing an unphysiological acylation motif also appears to undergo significant S-acylation in a second specific locus, the Golgi apparatus. Our results suggest that the substrate specificity of the plasma membrane S-acyltransferase(s) detected here may be rather broad, and that the plasma membrane and Golgi S-acylating activities are mediated by distinct S-acyltransferases.

Materials and Methods

Materials

[^{3}H]Palmitic acid (sp ac 40–80 Ci/mmol) was obtained from DuPont Canada (Mississauga, Ontario). Protected amino acids and other reagents for peptide synthesis were obtained from Novabiochem USA (La Jolla, CA) or Sigma Chemical Co. (St. Louis, MO). Cell culture media were purchased from GIBCO BRL (Burlington, Ontario). Tunicamycin (mixed isomers) was obtained from Sigma Chemical Co. and stored as a stock solution in serum-free medium at −80°C. BSA (fraction V, Sigma Chemical Co.) was fatty acid depleted by the procedure of Chen (12). Streptavidin (Sigma Chemical Co.) was labeled with Texas red (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

The mono-NBD- derivative of ethylenediamine was prepared by overnight reaction of NBD-chloride (100 mM) with five molar equivalents of ethylenediamine dihydrochloride and 1.05 equivalents of triethylamine in 3:1 (vol/vol) chloroform/methanol at room temperature. The reaction mixture was throroughly dried and chromatographed on a column of SP-Sephadex C-25 packed in water, eluting with a gradient of 0–30 mM aqueous NaCl to obtain pure mono–NBD-ethylenediamine hydrochloride, which was recovered by lyophilization. Fluorescent acylpeptides bearing the -edNBD group at the carboxyl terminus were then synthesized as described previously for analogous bimanylcysteamine-labeled acylpeptides (57, 80). Acylpentapeptides synthesized using standard Fmoc-based, solid-phase methods (20) were purified by flash chromatography, coupled to NBD-ethylenediamine using dicyclohexylcarbodiimide/hydroxybenzotriazole in dimethylformamide, then purified by preparative TLC on silica gel 60 as described previously (57, 80) both before and after O- and S-deprotection as appropriate. Purity of the final lipopeptide products was assessed by thin layer chromatography on both conventional and reverse-phase thin layer plates, visualizing both by fluorescence and by charring with sulfuric acid spray.

Methods

Cell Lines and Cell Culture. CV-1 and NIH-3T3 cells (obtained from Drs. Nahum Sonenberg and Philippe Gros, McGill University) and A431 cells (obtained from Dr. Maureen O'Connor-McCourt, NRC Biotechnology Research Institute, Montréal, Québec) were grown to 85–100% confluency in plastic culture dishes in DME supplemented with 5% FBS, gentamycin (50 μg/ml), and glutamine (10 mM). 3T3-L1 cells (obtained from Dr. Ralph Germinario, Lady Davis Institute, Jewish General Hospital, Montréal, Canada) were grown to 60–80% confluency in the same medium supplemented with 10% serum. Monolayers were washed three times with serum-free medium plus 5 mM pyruvate (SFM)[1] containing delipidated BSA (3 mg/ml), then three times with SFM alone before incubation with lipopeptides and/or [^{3}H]palmitate as described below.

For cell incubations lipopeptides (as 10 mM stock solutions in dimethylformamide) were diluted to 0.5 mM in SFM containing DTT (5 mM) plus either delipidated BSA (68 mg/ml) or, where indicated, sonicated POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) vesicles (3 mM) as a carrier. After incubation for 20 min at room temperature under argon, the mixtures were further diluted to the desired final lipopeptide concentrations (50 μM where not otherwise indicated) in SFM and immediately added to freshly washed cell monolayers. For incubations including [^{3}H]palmitate the labeled fatty acid was added to the final incubation mixture along with an additional 3 mg/ml delipidated BSA.

Fluorescence Assay of Lipopeptide S-Acylation. Washed cell monolayers in 100-mm culture dishes were incubated at 37°C with fluorescent lipopeptide (20 μM) in POPC vesicles (120 μM) in SFM. After incubation the cells were washed four times with SFM, then incubated for 15 min in 150 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 7.4, and harvested by trituration with a pasteur pipette (comparable results were obtained by scraping the cells from the plates without a 15-min preincubation). The cell suspension was pelleted (1,000 *g*, 5 min at 22°C), resuspended in 0.5 ml of the above buffer, chilled to 0°C, and partitioned in 4 ml (final vol) of 2:1:1 (vol/vol/vol) CH_2Cl_2/methanol/buffer acidified to pH ca 2 with dilute HCl. The lower phase and a second extract of the upper phase were pooled, washed once with 1:1 methanol/0.15 M NaCl, and dried under nitrogen before analysis by TLC (Whatman silica gel 60A plates, developing with 0.2% acetic acid, 5–12% methanol [depending on lipopeptide polarity] in CH_2Cl_2) alongside appropriate synthetic standards. After development fluorescent bands were moistened, scraped into methanol (3.5 ml), bath sonicated to ensure complete elution of the lipopeptide, and quantitated by fluorescence (absorption/emission wavelengths 390/472 nm, using a spectrofluorimeter [LS-5; Perkin-Elmer Corp., Norwalk, CT]) after pelleting the silica in a clinical centrifuge. In some experiments a portion of the total lipid/lipopeptide extract was taken for phospholipid assay as described previously (38).

S-Acylated lipopeptide samples for mass-spectrometric analysis were prepared from cells incubated with myrGCG-edNBD (20 mM, 37°C, 3 h) as described above, but the S-acylated lipopeptides were twice chromatographed on glass TLC plates to ensure a negligible background signal from organic contaminants. Fast atom bombardment mass spectrometry of these samples was carried out on a ZAB 2F HS instrument, using nitrobenzyl alcohol as the solvent and an instrumental resolution of 1/2,000. Appropriate mixtures of synthetic myrGC(acyl)G-edNBD standards in known proportions were chromatographed and analyzed in exactly the same manner to calibrate the relative sensitivity of mass-spectrometric detection for different S-acylated species.

[^{3}H]Palmitate Labeling of Cellular Components. Cell monolayers incubated at 37°C in 12-well culture dishes with [^{3}H]palmitate (with or without lipopeptide) were rapidly washed three times at room temperature with SFM plus 3 mg/ml delipidated BSA, then five times with 150 mM NaCl, 20 mM Hepes, pH 7.0. For assay of lipid and lipopeptide labeling the cells were then suspended by scraping in 0.4 ml of lifting buffer (250 mM sucrose, 20 mM sodium phosphate, 5 mM iodoacetamide, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml each leupeptin, aprotinin and soybean trypsin inhibitor, pH 7.4). The suspension was extracted at 0°C in CH_2Cl_2/methanol/acidified buffer as above. The washed extract was dried under nitrogen and analyzed by two-dimensional TLC on 10 × 10 cm PE Sil-G plates (Whatman), first adding to the extract either the authentic palmitoylated lipopeptide or 1-palmitoyl-2-pyrenedecanoyl PC (which comigrated with cellular PC) as a visual marker of the component(s) to be assayed for tritium incorporation. For assay of lipopeptide acylation the

1. *Abbrevations used in this paper*: POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SFM, serum-free DME supplemented with 5 mM pyruvate and 10 mM glutamine. Lipopeptides are designated using the one letter code plus these additional abbreviations: -edNBD, (2-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)ethyl)amino-; -caBim, (S-bimanylmercapto) ethylamine-; -(farn)-, S-farnesyl-; myr-, *N*-myristoyl-; -(palm)-, S-palmitoyl.

plates were developed in the first dimension with 0.2% acetic acid, 4.5–10% methanol (depending on the lipopeptide polarity) in CH_2Cl_2 and in the second dimension with 0.2% acetic acid in ethyl acetate (or in 50:50 diethyl ether/ethyl acetate for the least polar lipopeptides). For assay of phosphatidylcholine labeling the plates were developed in the first dimension with 50:20:10:10:2 (volume proportions) CH_2Cl_2/acetone/methanol/acetic acid/water and in the second with 65:25:1.5:1.5 CH_2Cl_2/methanol/conc. NH_4OH/water. In each case after developing and drying the plates the band localized by the fluorescent standard was moistened, recovered by scraping and incubated for 24 h in 10 ml of scintillation fluid (Cytoscint, ICN Canada, St. Laurent, Québec) before counting. In most cases the developed plates were also sprayed with En^{3}Hance (Dupont Canada) and visualized by autoradiography before scraping individual spots as above.

For assay of protein acylation, cells incubated with [^{3}H]palmitate were scraped into 250 μl of lifting buffer containing 0.5% Triton X-100, then mixed with 25 vol of 2:1 CH_2Cl_2/methanol and incubated for 40 min at 0°C. After centrifugation for 5 min in a clinical centrifuge the delipidated protein pellet (containing >95% of the input protein) was recovered, rinsed twice with 3 ml of cold 2:1 CH_2Cl_2/methanol, and freed of residual solvent under a gentle stream of nitrogen, then dissolved in 80 μl of 2.5% SDS at 60°C. Two 15-μl samples were withdrawn for protein assay (DC protein assay kit; BioRad Laboratories, Mississauga, Ontario, Canada), and the remaining 50 μl was combined with 12.5 μl of 100 mM DTT, 0.3125 M Tris, 12.5% glycerol, 0.05% bromophenol blue, pH 6.8, warmed to 60°C for 10 min, and analyzed by SDS-PAGE (34). Protein samples for autoradiography were analyzed using 10% polyacrylamide gels, which after electrophoresis were fixed for 30 min in 50% aqueous methanol/10% acetic acid, then soaked for 15 min in Amplify (Amersham Canada, Oakville, Ontario) before drying and autoradiography. Samples to be analyzed by scintillation counting were resolved using 12% gels; after electrophoresis the wet gels were cut into slices covering the range of molecular masses >10 kD for each lane. The slices were digested overnight in 1 ml of 1 M hydroxylamine, pH 7.0, then mixed with 15 ml of scintillation fluid and incubated for 24 h before counting.

Fluorescence Microscopy. CV-1 cells grown on glass coverslips to 80–90% confluency were washed with SFM for 37°C incubations. 4 and 15°C incubations were also carried out as described below, but using HBSS in place of SFM in all steps. The washed coverslips were incubated for the indicated times in 200 μl of SFM containing 50–100 μM lipopeptide, 0.5–1 mM sonicated POPC vesicles, and 0.5 mM DTT. The coverslips were then successively washed three times each with ice-cold SFM, 0.4 mM fatty acid–depleted albumin in SFM (incubating for 5 min each time) and SFM. Cells were then visualized using an inverted epifluorescence microscope (EM35; Carl Zeiss, Inc., Thornwood, NY) equipped with an MC100 camera.

For colocalization of biotinylated cell surface molecules and cell-associated myrGCG-edNBD, washed CV-1 cell monolayers on coverslips were first incubated with sulfosuccinimidyl-biotin (2 mg/ml) in PBS for 1 h at 4°C, then washed twice at 4°C with PBS, once with 50 mM glycine in PBS and twice with HBSS. The cells were then incubated with Texas red–labeled streptavidin (10 μg/ml) and myrGCG-edNBD (10 μM) for 3 h at 15°C, then SFM and albumin/SFM washed in the cold and examined using a confocal microscope (LSM 420; Carl Zeiss, Inc.). Digitized images were stored and printed without further modifications using a digital printer (XLS 8300; Eastman Kodak Co., Rochester, NY).

Results

Preliminary Characterization of Lipopeptide S-Acylation

In Fig. 1 are shown representative structures for some of the cysteinyl-containing lipopeptides used in this study. All of the fluorescent lipopeptides examined partitioned efficiently into lipid bilayers at submillimolar lipid concentrations and were shown to transfer rapidly ($t_{1/2} < 1$ min at 37°C) between and across bilayer membranes in fluorescence experiments using unilamellar lipid vesicles as model membranes as described previously (80). By virtue of these properties, when added to cultured mammalian cells (with serum albumin or lipid vesicles as a carrier), the lipopeptides rapidly gain access to intracellular as well as surface membranes, as could be demonstrated directly by fluorescence microscopy of cells incubated with nonmetabolized serinyl-lipopeptides such as myrGSG-edNBD (see below).

CV-1 cell monolayers incubated in serum-free medium with a fluorescent cysteinyl-containing lipopeptide such as myrGCG-edNBD (see Fig. 1) gradually accumulated the S-acylated form of the peptide. This process could be monitored using two complementary assays. In initial experiments, cells were incubated with the fluorescent lipopeptide alone. An organic solvent extract, containing the cell-associated lipopeptide and its derivatives as well as cellular lipids, was then prepared from the washed cells and analyzed by one-dimensional TLC as described in Materials and Methods. The resulting chromatograms revealed the presence of the unmodified lipopeptide, the S-acylated form (identified by its comigration with an authentic S-palmitoylated standard[2] and by its cleavage to the original cysteinyl-lipopeptide with neutral hydroxylamine) and a minor spot corresponding to the disulfide-linked lipopeptide dimer; no other fluorescent products were detected under these conditions. In a representative experiment, during a 2-h incubation at 37°C with 20 μM myrGCG-edNBD the cells accumulated 0.75 nmol of S-acylated lipopeptide, 0.10 nmol of unmodified lipopeptide, and 0.05 nmol of disulfide-oxidized lipopeptide dimer per mg of cellular protein (2.9, 0.4, and 0.2 nmol, respectively, per μmol total cellular phospholipid). Cells incubated with the alternative fluorescent-labeled lipopeptide myrGCG-caBim (Fig. 1) under otherwise identical conditions accumulated a similar amount of S-acylated lipopeptide (1.04 nmol/mg cellular protein), indicating that the S-acylation is not dependent on the presence of the NBD group. The lipopeptide myrGSG-edNBD was also taken up by the cells, but in this case no detectable fluorescent material other than the original lipopeptide was recovered.

Incubations of cells with lipopeptides were normally carried out in the presence of a low concentration of DTT (0.5 mM), which was required to minimize the oxidation of cysteinyl-lipopeptides during extended incubations. However, in control experiments we found that DTT was not required for efficient lipopeptide S-acylation during short incubations (0–30 min), where lipopeptide oxidation was limited, and did not significantly affect either cellular morphology or the S-acylation of cellular proteins under the conditions used here.

In subsequent experiments, to increase the sensitivity of the lipopeptide S-acylation assay and the number of samples that could be simultaneously examined, we modified the above assay by coincubating cells with lipopeptide and tritiated palmitic acid. An organic solvent extract was then prepared as above, mixed with the appropriate S-palmitoylated lipopeptide standard and separated by two-dimensional TLC as described in Materials and Methods. For all monocysteinyl-lipopeptides tested autoradiography of the

2. The S-acylated lipopeptides recovered from CV-1 cells comigrated with chemically prepared standards bearing long chain S-acyl groups (palmitoyl, stearoyl, or oleoyl, which were not resolved in our chromatographic systems) but showed significantly higher mobilities than standards bearing shorter S-acyl groups such as acetyl. This result, which was observed for all lipopeptide substrates examined, is consistent with our direct mass-spectrometric demonstration (described later in this section) that myrGCG-edNBD is modified with palmitoyl, stearoyl, and oleoyl chains.

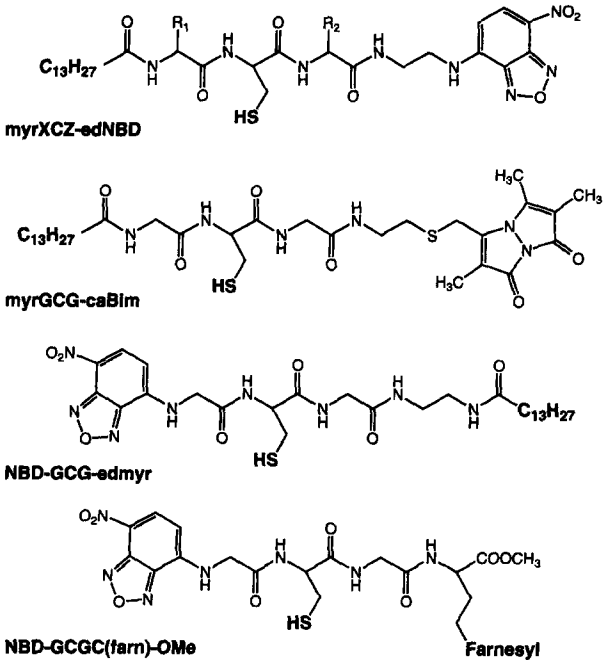

Figure 1. Structures of the fluorescent lipopeptides used in this study.

resulting TLC chromatogram revealed a unique novel radiolabeled band, comigrating exactly with the S-palmitoylated lipopeptide standard, as illustrated in Fig. 2 *A* for CV-1 cells incubated with myrGCG-edNBD. Under the TLC conditions used the S-acylated lipopeptide was in all cases cleanly resolved from other radiolabeled bands present, allowing reliable quantitation of the formation of S-[^{3}H]acyl lipopeptide. Under the same conditions cells incubated with the serinyl-lipopeptide myrGSG-edNBD showed negligible incorporation of radioactivity into material migrating at the position of myrGS(palm)G-edNBD (Fig. 2 *B*). The lipopeptide acylation process observed here thus appears to be highly selective for modification of cysteine over serine residues.

In additional experiments we examined further basic characteristics of the lipopeptide S-acylation reaction described above, using CV-1 cells with myrGCG-edNBD as the lipopeptide substrate. As illustrated in Fig. 3 *A*, formation of radiolabeled S-acylated lipopeptide proceeds over a time course of at least several hours, with no detectable initial lag even when the lipopeptide and [^{3}H]palmitate are added to the cells simultaneously. This latter result suggests that the pool(s) of acyl-donor molecules for the lipopeptide S-acylation reaction rapidly becomes labeled to essentially constant specific activity upon addition of exogenous [^{3}H]palmitate. In agreement with this conclusion, the extent of formation of radiolabeled myrGC(acyl)G-edNBD during a 1-h coincubation of CV-1 cells with [^{3}H]palmitate plus myrGCG-edNBD was not detectably enhanced by preincubating the cells with the labeled palmitate for up to 2 h before lipopeptide addition (not shown). Pulse-chase experiments, in which cells were continuously incubated with myrGCG-edNBD (50 μCi), first with [^{3}H]palmitate (50 μCi/ml, 1 h) and then, after repeated washings, with unlabeled palmitate (500 μM), demonstrated turnover of the labeled S-acylated lipopeptide with a half-time of roughly 4 h (not shown). As shown in Fig. 3 *B*, when CV-1 cell monolayers are incubated for 2 h with [^{3}H]palmitate (50 μCi/ml) and varying concentrations of myrGCG-edNBD, formation of radiolabeled S-acylated lipopeptide increases in a apparently saturable manner as a function of the added lipopeptide concentration. Based on the above results further experiments were carried out using a standard lipopeptide concentration of 50 μM and an incubation time of 2 h except where otherwise indicated. These conditions allowed accurate quantitation of S-acylation even for lipopeptides that were modified much less efficiently than myrGCG-edNBD.

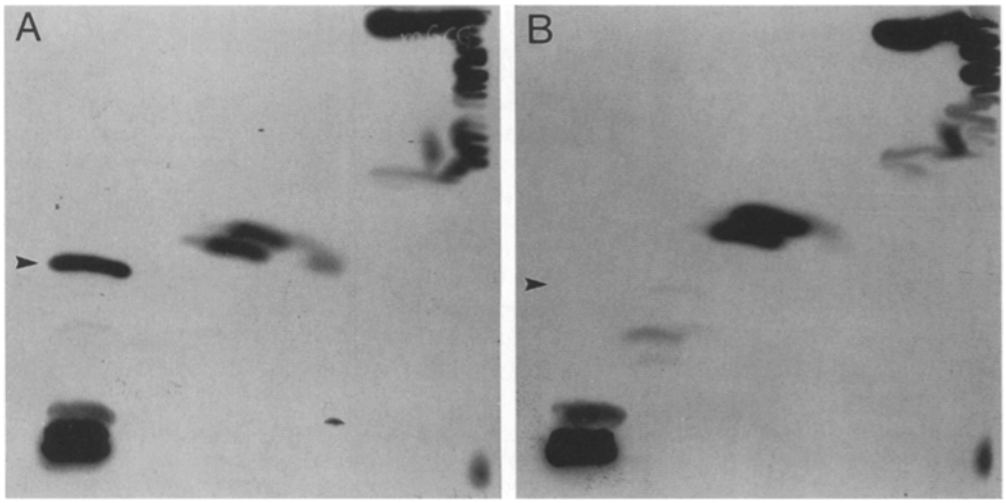

Figure 2. Autoradiogram of TLC chromatograms of lipid/lipopeptide samples from CV-1 cells incubated for 2 h at 37°C with [^{3}H]palmitate (50 μCi/ml) plus either (*A*) 50 μM myrGCG-edNBD or (*B*) 50 μM myrGSG-edNBD. Samples were applied as a 1-cm band at the origin to optimize resolution in the first dimension. The band marked with an arrowhead in *A* comigrated exactly with authentic myrGC(palm)G-edNBD added to the sample before chromatography. The minor bands migrating around the diagonal at R_f = ca 0.2 in chromatogram (*B*) were also detected in chromatogam (*A*) upon longer exposures. No labeled band was observed in chromatogram (*B*) at the position of either myrGS(acyl)G-edNBD (*arrowhead*) or myrGC(acyl)G-edNBD, even after longer exposures. Other experimental details were as described in Materials and Methods.

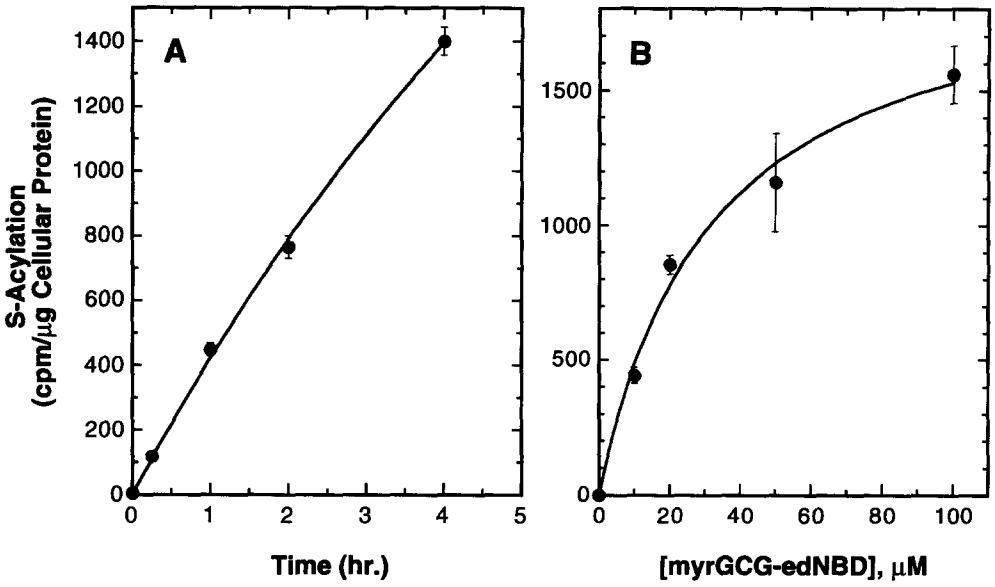

Figure 3. (*A*) Time course of incorporation of [^{3}H]palmitate into myrGC(acyl)G-edNBD by CV-1 cells incubated with myrGCG-edNBD (50 μM) and [^{3}H]palmitate (50 μCi/ml) at 37°C. (*B*) Lipopeptide concentration dependence of the incorporation of [^{3}H]palmitate into myrGC(acyl)G-edNBD by CV-1 cells during a 2-h incubation at 37°C. In both panels, values shown represent the mean (± SD) of three determinations at each time point using separately incubated cell samples. Time courses determined in replicate experiments were very similar in form to that shown but varied somewhat in the absolute magnitude of lipopeptide-incorporated counts at any given time/concentration. Other experimental details were as described in Materials and Methods.

S-Acylation by CV-1 cells of exogenous myrGCG-edNBD and several other lipopeptides tested was readily detected, using the fluorescence-based assay described above, when the incubation temperature was reduced from 37 to 15°C or even to 4°C, although the rate of S-acylation was significantly reduced at the latter temperatures (not shown). However, by suitably prolonging the time of incubation of cells with lipopeptides at these lower temperatures, the S-acylated form of the lipopeptides could be accumulated to levels comparable to those observed at 37°C.

The nature of the S-acyl groups incorporated into myrGC(acyl)G-edNBD by CV-1 cells was examined by mass spectrometry. Cells were incubated for 3 h with 20 μM myrGCG-edNBD (without tritiated palmitate), and the S-acylated lipopeptide was extracted and purified by TLC as above. Fast atom bombardment mass-spectrometric analysis of the isolated material, combined with parallel analyses of appropriate synthetic S-acylated standards, identified peaks with masses corresponding to the S-palmitoyl (77%), S-stearoyl (15%), and S-oleoyl (8%) forms, in the relative molar proportions indicated. No other type of S-acyl substituent could be identified above the detection threshold (estimated as 2–3 mol% of total lipopeptide-coupled S-acyl chains).

To determine whether the S-acylation of cysteinyl-lipopeptides might be peculiar to CV-1 cells, using the [^{3}H]palmitate-labeling assay we compared the S-acylation of myrGCG-edNBD by four cell lines: CV-1 (monkey kidney fibroblast) cells, the murine fibroblast line NIH-3T3, the human epidermoid carcinoma cell line A431, and the preadipocytic murine cell line 3T3-L1. As shown in Table I, all of these cell lines showed substantial S-acylating activity toward myrGCG-edNBD but virtually no activity (>10^3-fold less) toward the analogous serinyl lipopeptide. While the values shown in Table I cannot be assumed to reflect quantitatively the relative S-acylating activities of the different cell lines (given the possibility of differences in the specific activities of the relevant [^{3}H]acyl-donor pool[s] between cell lines), it is clear that these cell types are at least qualitatively similar in their abilities to acylate selectively the cysteinyl, but not the serinyl lipopeptide.

Comparison of S-Acylation of Exogenous Lipopeptides and Cellular Proteins

We have previously shown (57) that in model systems cysteinyl-containing lipopeptides like those examined here can acquire S-acyl groups by nonenzymatic trans-S-acylation reactions from other long-chain acyl thioesters, including S-acylpeptides, when both species are present in the same lipid bilayer. It is thus conceivable in principle that lipopeptides such as myrGCG-edNBD could acquire S-acyl groups by nonenzymatic exchange from S-acylated membrane proteins. To determine whether this mecha-

Table I. Incorporation of [^{3}H]Palmitate into myrGC(acyl)G-edNBD versus myrGS(acyl)G-edNBD by Different Mammalian Cell Lines

	Incorporation of [^{3}H]palmitate (cpm/μg cellular protein) into:	
Cell line	myrGC(acyl)G-edNBD	myrGS(acyl)G-edNBD
CV-1	2,262 ± 60	1.6 ± 0.2
A431	2,161 ± 124	3.3 ± 0.9
NIH-3T3	4,777 ± 838	4.8 ± 2.2
3T3-L1	6,645 ± 1076	5.1 ± 2.3

Cell monolayers were incubated for 2 h at 37°C with myrGCG-edNBD (50 μM) plus [^{3}H]palmitate (50 μCi/ml) and subsequently analyzed for formation of [^{3}H]-labeled S-acylated lipopeptide as described in Materials and Methods. Values shown are means (± SD) of determinations for triplicate cell samples incubated in parallel in one experiment; a duplicate experiment gave a similar pattern of results but ~25% higher labeling of the lipopeptides.

nism could represent a major pathway for the S-acylation of exogenous cysteinyl lipopeptides, we compared the extent and kinetics of S-[^{3}H]acylation of the endogenous proteins of CV-1 cells versus added myrGCG-edNBD.

CV-1 cells readily incorporated radiolabel from exogenous [^{3}H]palmitate into cellular proteins, as demonstrated by SDS-PAGE and autoradiography of total cellular proteins (Fig. 4, lane *3*). The right-most lane of Fig. 4 represents a parallel sample (containing an equal amount of protein applied to the gel) that was treated with neutral hydroxylamine (16 h, 22°C) before electrophoresis. The almost complete disappearance of all major labeled bands upon hydroxylamine treatment (89–92% of total protein-associated counts, determined by scintillation counting of three independent samples) suggests that at most a small fraction of the incorporated label represents (hydroxylamine-resistant) amide-linked *N*-[^{3}H]myristoyl groups, which in principle could arise via catabolism of [^{3}H]palmitic acid.

To quantitate [^{3}H]palmitate labeling of cellular proteins, solvent-delipidated proteins were separated from residual lipids and other low molecular weight contaminants by SDS-PAGE, and protein-linked ^{3}H-acyl groups were quantitated by liquid scintillation counting as described in Materials and Methods. In Fig. 5 we compare the measured extents of radiolabel incorporation into cellular proteins, S-acylated lipopeptide, and phosphatidylcholine, a major cellular phospholipid, when CV-1 cells were incubated for 2 h at 37°C with [^{3}H]palmitate (200 μCi/ml) and 50 μM myrGCG-edNBD. As shown in this figure, during such incubations the incorporation of radiolabel into the S-acylated lipopeptide exceeds that into cellular proteins by over tenfold and in fact is almost 20% as large as the incorporation of radiolabel into phosphatidylcholine. While the presence of the lipopeptide significantly reduces protein ^{3}H-acyl labeling in such experiments (Fig. 6 *A*, *solid circles*), the magnitude of this effect is only approximately twofold at the 50 μM lipopeptide concentration used above. Pulse-chase experiments, in which cells were labeled for 1 h with 200 μCi/ml [^{3}H]palmitate, washed, and then chased with unlabeled palmitate (500 μM), showed, moreover, no significant effect of myrGCG-edNBD (50 μM) on the overall rate of turnover of total protein-bound ^{3}H-acyl groups (not shown). These results collectively suggest that S-acylated cellular proteins do not constitute a sufficiently large reservoir of ^{3}H-acyl groups to serve as a major donor of such groups for S-acylation of myrGCG-edNBD.

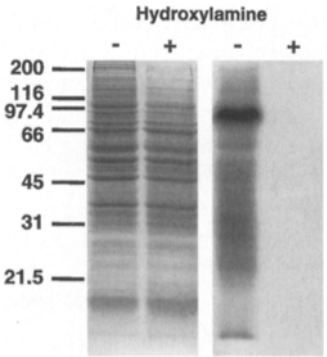

Figure 4. SDS-PAGE analysis of total cellular proteins from CV-1 cells incubated with [^{3}H]palmitate (200 μCi/ml) for 2 h at 37°C. Left lanes, Coomassie staining profiles of samples incubated for 16 h at room temperature either with 1 M Tris, pH 7.0 (−), or 1 M hydroxylamine, pH 7.0 (+), containing 0.1% SDS. Right lanes, autoradiograms of replicate aliquots of the same samples. Migration positions of protein standards of known molecular mass (in kD) are shown at the left. After Tris or hydroxylamine treatment protein samples were twice precipitated with cold 2:1 CH_2Cl_2/methanol (giving >90% final recovery of total protein) before SDS-PAGE analysis. Protein assay of loaded samples and scanning densitometry of the stained gels confirmed that equal amounts of protein were loaded for Tris- and hydroxylamine-treated samples.

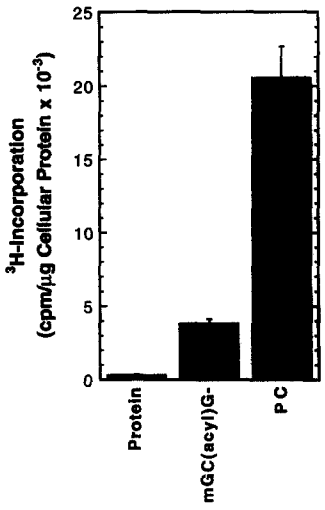

Figure 5. Comparison of ^{3}H incorporation into total cellular protein, myrGC(acyl)G-edNBD (mGC(acyl)G-), and phosphatidylcholine (*PC*) by CV-1 cells incubated for 2 h with [^{3}H]palmitate (200 μCi/ml) and myrGCG-edNBD (50 μM) at 37°C. Labeling of protein, lipopeptide, and phosphatidylcholine were determined as described in Materials and Methods. Values shown represent the mean (± SEM) for four independent experiments, each using three separately incubated cell samples.

The conclusion just noted is supported by comparing the time courses of ^{3}H-acyl labeling of cellular proteins and of exogenous myrGCG-edNBD when CV-1 cells are incubated with [^{3}H]palmitate (200 μCi/ml). As shown in Fig. 6 *B*, upon addition of labeled palmitate total labeling of cellular proteins increases progressively over several hours without or (not shown) with myrGCG-edNBD present. Autoradiography of ^{3}H-acylated cellular proteins (not shown) revealed no major protein bands that reached a constant level of ^{3}H incorporation in less than 2–4 h after addition of [^{3}H]palmitate. As noted above, the incorporation of label into myrGC(acyl)G-edNBD upon addition of labeled palmitate proceeds at a steady rate, with no detectable initial lag when the lipopeptide and [^{3}H]palmitate are added to the cells simultaneously (Fig. 3 *B*) and with no enhancement of the rate of [^{3}H]palmitate incorporation when the labeled fatty acid is added up to 2 h before adding the lipopeptide. These findings appear inconsistent with a hypothetical precursor–product relationship between S-acylated cellular proteins and the S-acylated lipopeptide.

The above results effectively rule out the possibility that exogenous cysteinyl lipopeptides such as myrGCG-edNBD acquire S-acyl groups primarily by nonspecific transacylation reactions from S-acylated cellular proteins. Similar results were obtained in parallel experiments using other lipopeptides discussed below (not shown), including species that were S-acylated considerably less efficiently than myrGCG-edNBD.

The antibiotic tunicamycin, first characterized as an inhibitor of protein glycosylation, has also been shown to inhibit protein S-acylation in cultured neuronal cells at relatively high concentrations (53). It was therefore of interest to compare the effects of tunicamycin on the S-acylation by CV-1 cells of cellular proteins and of myrGCG-edNBD. As shown in Fig. 7, tunicamycin inhibited the S-acy-

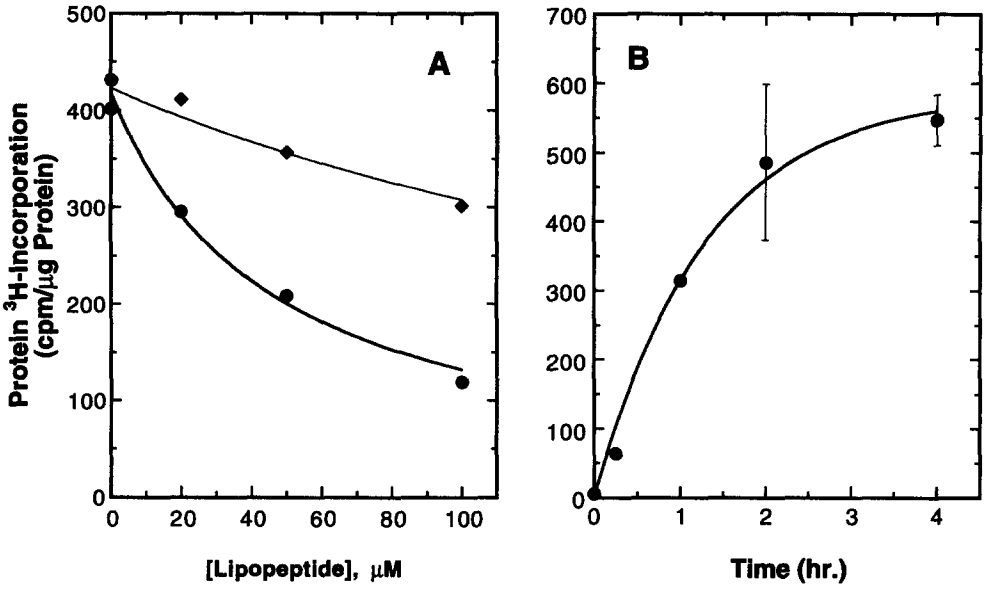

Figure 6. (*A*) Effect of myrGCG-edNBD (*filled circles*) or myrGSG-edNBD (*filled diamonds*) on [^{3}H]palmitate labeling of total proteins from CV-1 cells. Cells were incubated for 2 h at 37°C with [^{3}H]palmitate (200 μCi/ml) and the indicated concentrations of myrGCG-edNBD, then analyzed for ^{3}H labeling of total cellular proteins as described in Materials and Methods. (*B*) Time course of [^{3}H] labeling of total cellular proteins by CV-1 cells incubated with [^{3}H]palmitate (200 μCi/ml) at 37°C. Data shown represent the mean (± half-range) determined for duplicate cell samples, incubated separately with labeled palmitate, at each time point in a representative experiment. Replicate experiments gave comparable patterns of results but varied somewhat in the absolute levels of protein labeling.

lation of both types of acceptor molecule over a similar range of concentrations, albeit with moderately greater potency for inhibition of lipopeptide S-acylation. The fact that the inhibition profiles are not identical may not be surprising, as protein S-acylation may occur in multiple compartments and will reflect the modification of newly synthesized proteins as well as of proteins undergoing S-acyl turnover.

Structural Specificity of Lipopeptide S-Acylation

In the experiments summarized in Fig. 8, we compared the ability of CV-1 cells to S-acylate cysteinyl-containing lipopeptides of varying sequence and structure. To this end CV-1 cells were coincubated for 2 h at 37°C with tritiated palmitate (50 μCi/ml) and different lipopeptides (50 μM) in serum-free medium, and formation of radiolabeled S-acylpeptide was subsequently assayed as described above. In control experiments we found that the relative efficiencies of S-acylation of several different lipopeptides tested under the above conditions reflected faithfully the relative initial S-acylation rates measured for these species over shorter time courses (not shown). To ensure that different lipopeptides would partition to similar concentrations into cellular membranes (in competition with the lipopeptide carrier) in these experiments we used as carrier POPC vesicles, which should bind the lipopeptides in a manner similar to the lipid bilayers of membranes. In each experiment the extent of S-acylation of a given lipopeptide was normalized to the cellular protein content and to the level of S-acylation of myrGCG-edNBD measured in parallel incubations, to correct for variations between experiments in cell number and/or in the specific activity of the cellular pool(s) of the [^{3}H]acyl donor(s) for the lipopeptide S-acylation reaction.

As the data shown in Fig. 8 demonstrate, a variety of cysteinyl lipopeptides can be S-acylated by CV-1 cells, although the rate of acylation varies substantially with the structure of the lipopeptide. Taking as a reference the lipopeptide myrGCG-edNBD, it can be seen from Fig. 8 *A* that increasing the bulk of the amino acid side chain at position 1 consistently decreases the efficiency of acylation, most markedly when the side chain is branched at the beta-carbon. Nonetheless, significant S-acylation is observed even for lipopeptides bearing bulky residues such as Ile, Val, or Phe at the myristoylated NH_2-terminal position. Substitution of glycine by leucine, valine, or threonine at the lipopeptide 3 position also decreases somewhat the efficiency of S-acylation. Interestingly, the lipopeptides myrCG-edNBD and myrC-edNBD, in which the modifiable cysteinyl residue is itself N-myristoylated, were acylated with significantly greater efficiencies than was myrGCG-edNBD (Fig. 8 *B*). Evidence discussed below suggests that myrCG- and myrC-edNBD are substrates for an additional S-acylating activity that does not efficiently modify the other lipopeptides discussed above. The serinyl lipopeptides myrSG-edNBD and myrS-edNBD (not shown) showed essentially no ^{3}H labeling (>200-fold less than for myrCG- or myrC-edNBD) in parallel incubations.

The S-acylation of several NH_2-terminally myristoylated pentapeptides was examined in parallel with the above studies of the modification of myristoylated tripeptides (Fig. 8 *C*). The lipopeptide myrGCGCS-edNBD, representing the NH_2-terminal sequence of the human nonre-

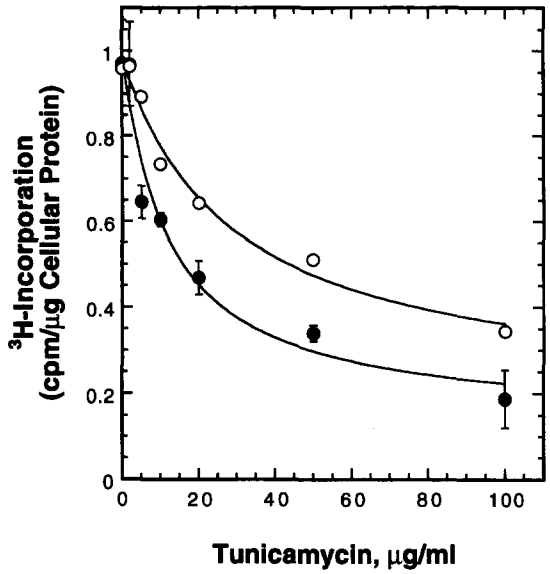

Figure 7. Tunicamycin inhibition of ^{3}H incorporation into total cellular proteins (*open circles*) or myrGCG-edNBD (*closed circles*) by CV-1 cells incubated for 2 h at 37°C with [^{3}H]palmitate (200 μCi/ml) and myrGCG-edNBD (50 μM). For clarity of presentation the curves for inhibition of lipopeptide and protein S-acylation have been scaled to pass through a common y-intercept; actual ^{3}H-labeling of lipopeptide (per μg of sample protein) was roughly 15-fold higher than protein labeling at zero tunicamycin. Data shown are from a representative experiment using triplicate (independently incubated) samples for determination of lipopeptide acylation and single samples for determination of protein acylation. Other details were as described in Materials and Methods.

ceptor tyrosine kinase p56^{lck}, was recovered from CV-1 cells in both mono- and diacylated forms (52 ± 4% and 48 ± 4% of total S-acylated lipopeptide, respectively, in nine determinations). Unfortunately, this lipopeptide showed an unusually fast rate of oxidation in solution even in the presence of DTT, so that the measured efficiency of S-acylation shown for this species in Fig. 8 *C* is artifactually diminished. The efficiency of S-acylation of the lipopentapeptide myrGCGSS-edNBD was similar to that of myrGCG-edNBD, while that for myrGSGCS-edNBD was approximately twofold lower. The lipopeptide myrGCTLS-edNBD, representing the NH_2-terminal sequence of the heterotrimeric G protein subunit $G_{\alpha i3}$, was also readily acylated by CV-1 cells, albeit with a somewhat lower efficiency than were the other lipopentapeptides examined.

In Fig. 8 *D* are summarized data describing the effect of the hydrophobic 'anchor' group on the S-acylation of a series of NBD-labeled lipopeptides containing the core sequence -GCG-. Within a homologous series of lipopeptides (acyl)-GCG-edNBD, members with N-acyl chain lengths from 10 to 16 carbons were S-acylated with efficiencies that varied over a range of only some twofold. By contrast, acetyl-GCG-edNBD was acylated with an efficiency over 100-fold lower than that observed for the corresponding *N*-myristoyl species. These results suggest that the ability of the *N*-acyl group to promote membrane partitioning may be more important than its specific structure in determining the suitability of these cysteinyl-lipopeptides as acylation substrates.[3] In agreement with this conclusion, the 'inverted' lipopeptide NBD-GCG-ed-myristoyl and the farnesylated peptide NBD-GCGC(farn)-OMe (see Fig. 1) were both found to be S-acylated with efficiencies similar to those observed for myrGCG-edNBD (Fig. 8 *D*).

Fluorescence-Microscopic Localization of S-Acylating Activity

As noted above, in their unmodified forms the fluorescent lipopeptides employed in this study transfer rapidly between and across bilayer membranes. By contrast, as we have recently demonstrated (76), in their S-acylated (doubly lipid-modified) forms such lipopeptides exhibit extremely slow rates of spontaneous interbilayer exchange. As a result, fluorescent cysteinyl lipopeptides are expected to accumulate preferentially in the membrane compartment(s) where they become S-acylated, permitting these compartments to be visualized by fluorescence microscopy. The specificity of fluorescence labeling of such compartments can be enhanced by repeatedly washing the cells at 4°C with albumin-containing solutions, which were shown to extract the (diffusible) non–S-acylated form of the lipopeptides efficiently (>90%) and selectively, and by carrying out the acylation reactions at reduced temperatures (4 or 15°C) where a number of processes of intercompartmental vesicular transport of proteins and lipids are strongly suppressed (55).

As shown in Fig. 9 *a*, CV-1 cells incubated for 4 h at 15°C with myrGCG-edNBD (50 μM) show pronounced selective labeling of the plasma membrane. Similar prominent labeling of the plasma membrane was observed for cells incubated with this lipopeptide at 4 or at 37°C (not shown). Strong preferential labeling of this membrane was observed as well in cells not washed with albumin before microscopic examination, although in this case weak labeling of intracellular membranes could also be seen (not shown).

To confirm that the structure prominently labeled by myrGCG-edNBD was in fact the plasma membrane, cells were surface biotinylated with sulfosuccinimidyl biotin at 4°C, then incubated with lipopeptide for 4 h at 15°C, washed repeatedly with cold albumin-containing SFM, and finally decorated at 4°C with Texas red–labeled streptavidin. The doubly-labeled cells were then examined by confocal microscopy immediately after warming to room temperature, giving the results illustrated in Fig. 9, *c* and *d*. The very close correspondence of the distributions of S-acylated lipopeptide (Fig. 9 *c*) and surface-bound Texas red–streptavidin (Fig. 9 *d*) confirm that the S-acylated lipopeptide is generated predominantly if not exclusively in the plasma membrane under these conditions. A

3. It may appear surprising, in the light of the results of Peitzsch and McLaughlin (54), that an N-acyl group as short as 10 carbons could still support significant membrane binding of an N-acylpeptide like those examined here. However, the NBD group itself also appears to contribute modestly to the net affinity of these lipopeptides for lipid surfaces, as was observed previously for NBD-labeled phospholipids (47; Silvius, J., unpublished results). We have found in control experiments carried out as described previously (80) that even *N*-decanoyl-GCG-edNBD exhibits significant affinity for binding to lipid bilayers at the effective membrane–lipid concentrations prevailing within a typical cell (several mM [23]), while the N-acetylated species does not.

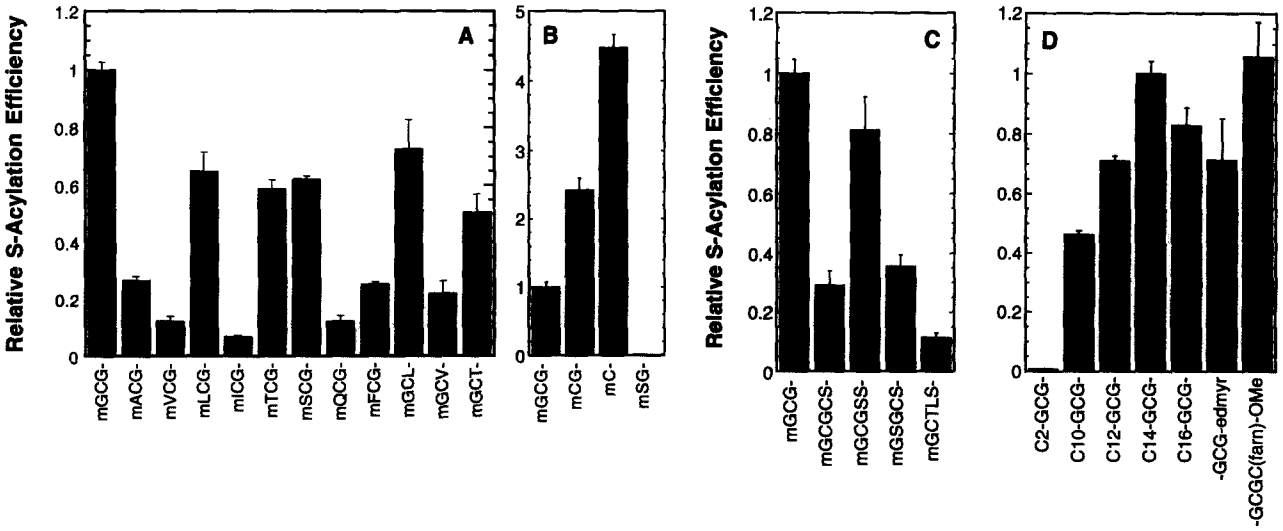

Figure 8. Relative efficiencies of S-acylation of lipopeptides of different structure and sequence. CV-1 cells were incubated for 2 h at 37°C with [^{3}H]palmitate (50 μCi/ml) and the indicated lipopeptides, then harvested and analyzed for [^{3}H]palmitate incorporation into S-acyl lipopeptide as described in Materials and Methods. For each experiment the extent of ^{3}H incorporation into each (S-acylated) lipopeptide was normalized to sample protein content and to the mean level of ^{3}H incorporation into myrGC(acyl)G-edNBD measured in parallel incubations. Data shown represent the mean (± SEM) determined using triplicate samples in each of a minimum of three independent experiments.

small amount of vesicular fluorescence can be observed intracellularly for both the S-acylated lipopeptide and the labeled streptavidin in Fig. 9, *c* and *d*, reflecting a small amount of internalization of plasma membrane that was observed during the limited time (~5 min) that the cells were exposed to room temperature during microscopic visualization. As vesicular transport of materials from the *trans*-Golgi to the plasma membrane has been shown to be strongly inhibited at temperatures ≤15°C (13, 29, 40, 50, 67, 79), the above results strongly implicate the plasma membrane itself as the site of S-acylation of myrGCG-edNBD.

As illustrated in Fig. 9 *b*, the S-acylated derivatives of the lipopeptides myrGCT- and myrACG-edNBD also showed preferential accumulation in the plasma membrane under the above incubation conditions. The lipopeptides myrGCGCS-, myrGCGSS-, myrGSGCS-, myr GCTLS-, myrGCL-, myrQCG-, myrSCG-, myrVCG- and myrLCG-edNBD, decanoyl-LCG-edNBD, NBD-GCGC (farn)-OMe, and the inverted lipopeptide NBD-GCG-edmyristoyl were also examined as above and all showed preferential localization to the plasma membrane under these conditions (not shown).

In contrast to the behavior of the cysteinyl-containing lipopeptides just discussed, cells labeled with the lipopeptide myrGSG-edNBD (without subsequent albumin washing, which removed the intracellular fluorescence) showed widespread staining of a variety of intracellular membrane compartments and only weak staining of the plasma membrane, at either 15°C (Fig. 9 *e*) or 37°C (not shown). This pattern of staining is consistent with that expected given that this lipopeptide can readily diffuse between and across cellular membranes but does not become O-acylated (and hence trapped) in any cellular compartment. The pattern of staining observed with myrGSG-edNBD (or with myrGSGSS-edNBD [not shown]) is similar to that observed for cells incubated with the fluorescent phospholipid C_6-NBD-PA (Fig. 9 *f*), which has been previously shown to be rapidly converted to fluorescent diacylglycerol at the cell surface and subsequently to become incorporated into a variety of cellular membranes (49).

While a variety of cysteinyl-containing lipopeptides were preferentially accumulated (as their S-acyl derivatives) in the plasma membrane, the lipopeptides myrC-edNBD and myrCG-edNBD gave strong labeling not only of the plasma membrane but also of a distinct, perinuclear compartment as illustrated in Fig. 10. This pattern of cell labeling was observed for both lipopeptides at 37°C (illustrated in Fig. 10 *a* for myrC-edNBD) and at 15°C (Fig. 10, *c* and *e*). MyrC-edNBD also gave a similar pattern of cell labeling at 4°C, although myrCG-edNBD gave less distinct perinuclear labeling at this temperature. By contrast, the serinyl-lipopeptides myrS-edNBD (Fig. 10 *b*) and myrSG-edNBD (not shown) labeled a variety of cellular membranes, with no evident preferential labeling of either the plasma membrane or the perinuclear compartment just noted either at 15 or 37°C. This result suggests that the addition of a second acyl chain (which as shown above does not occur for the serinyl lipopeptides) is essential for the preferential localization of species such as myrC- and myrCG-edNBD to the perinuclear compartment as well as to the plasma membrane.

To identify the perinuclear compartment labeled by myrCG-edNBD and myrC-edNBD, cells were simultaneously incubated with one of these lipopeptides and with C_8-DECA-ceramide (*N*-[[[8-[7-diethylamino]coumarin-3-yl]carbonyl]amino]octanoylsphingosine), which like other fluorescent ceramides preferentially labels the Golgi apparatus, particularly at reduced temperatures (33, 36, 50). The strong colocalization of the two probes within the perinuclear structures after coincubation at 15°C (Fig. 10, *c–f*), as well as at 37°C or, for myrC-edNBD, 4°C (not

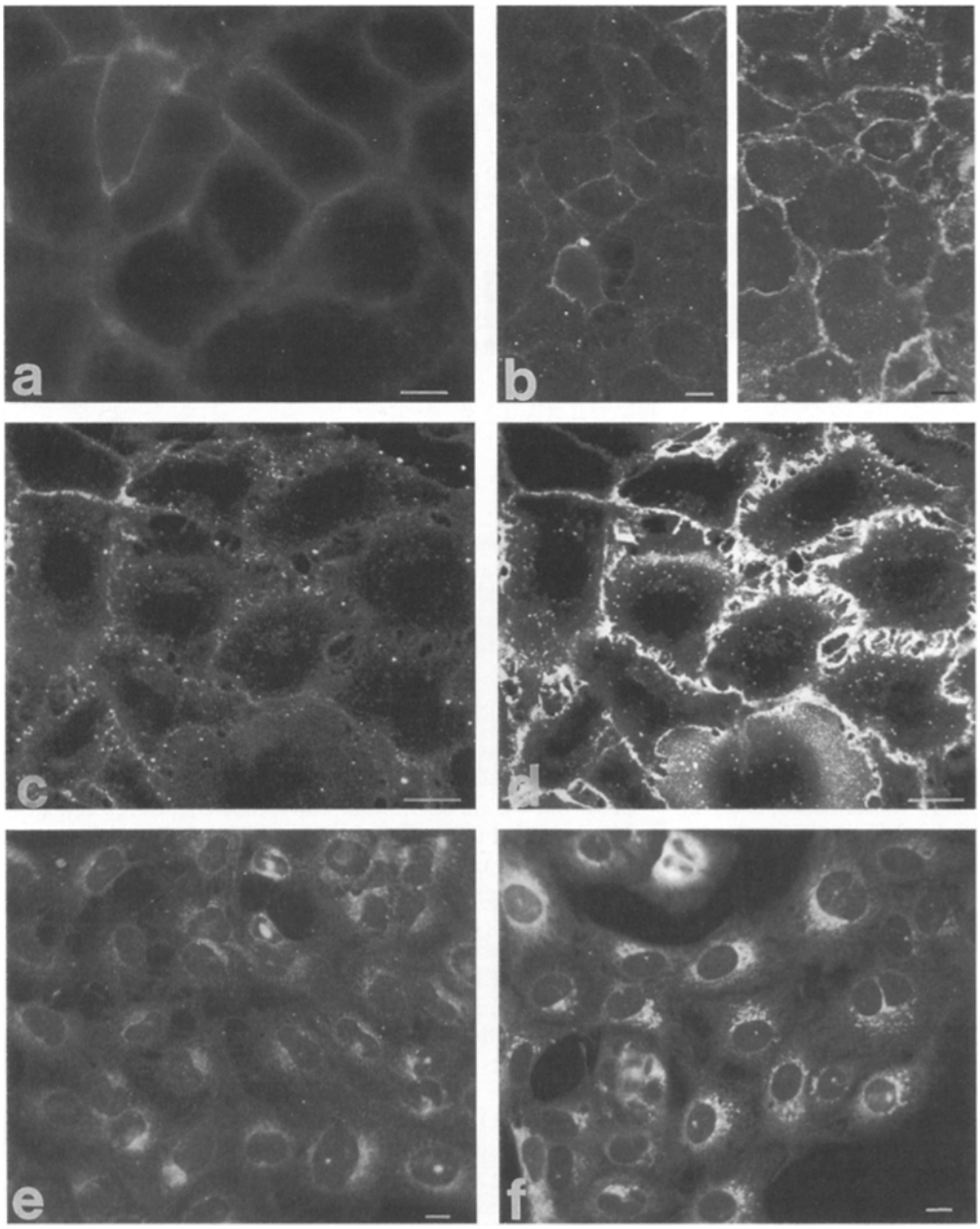

Figure 9. Fluorescence-microscopic localization of the intracellular site(s) of S-acylation of lipopeptides in CV-1 cells. *a–d* represent cell samples incubated for 4 h at 15°C with lipopeptides (50 μM), then washed with albumin at 4°C to remove unmodified lipopeptide as detailed in Materials and Methods. (*a* and *b*) Conventional microscopic images of cells incubated with (*a*) myrGCG-edNBD or (*b*) myrGCT-edNBD (*left half panel*) or myrACG-edNBD (*right half panel*). (*c* and *d*) Confocal microscopic images of surface-biotinylated cells incubated with myrGCG-edNBD, then washed with albumin/SFM, decorated with Texas red–labeled streptavidin at 4°C and finally visualized immediately after rewarming to room temperature: (*c*) NBD-labeled lipopeptide image; (*d*) Texas red–streptavidin image. (*e* and *f*) Conventional microscopic images of cells incubated for 2 h at 15°C with (*e*) myrGSG-edNBD (50 μM) or (*f*) C_6-NBD-PA (50 μM, using sonicated POPC vesicles [350 μM] as carrier). Bar, 10 μm.

shown) confirms that the second compartment labeled by myrC(acyl)-edNBD and myrC(acyl)G-edNBD is the Golgi apparatus. Previous studies (37, 67, 73) have suggested that vesicular transport of materials from the ER to the Golgi is effectively suppressed at temperatures ≤15°C. The absence of discernible reticular staining and the strong Golgi labeling in cells incubated with myrC- and myrCG-edNBD at 15°C (and with myrC-edNBD at 4°C) suggests that these lipopeptides accumulate in the Golgi through S-acylation in this compartment itself, rather than through S-acylation in the endoplasmic reticulum followed by bulk membrane transport to the Golgi. This conclusion can be compared with previous results which suggest that S-acylation of various integral membrane proteins occurs posttranslationally in the *cis*-Golgi or in an immediately preceding compartment along the secretory pathway (8, 58, 71). The observation that both lipopeptides also accumulate in the plasma membrane even at 4°C or 15°C, where vesicular transport from the Golgi to the plasma membrane is strongly suppressed (13, 29, 40, 50, 67, 79), suggests that the plasma membrane is also a bona fide site of S-acylation of these species.

Discussion

The cysteinyl lipopeptides used in this study offer several significant potential advantages as model substrates to examine in situ the S-acylation of cysteinyl-containing sequences (including, though not necessarily limited to, those occurring in N-acylated or prenylated proteins) in mammalian cells. First, the uncharged lipopeptides, being diffusible and membrane permeant, can be incorporated into cells under readily controlled conditions. Second, the S-acylated forms of the lipopeptides can readily be sepa-

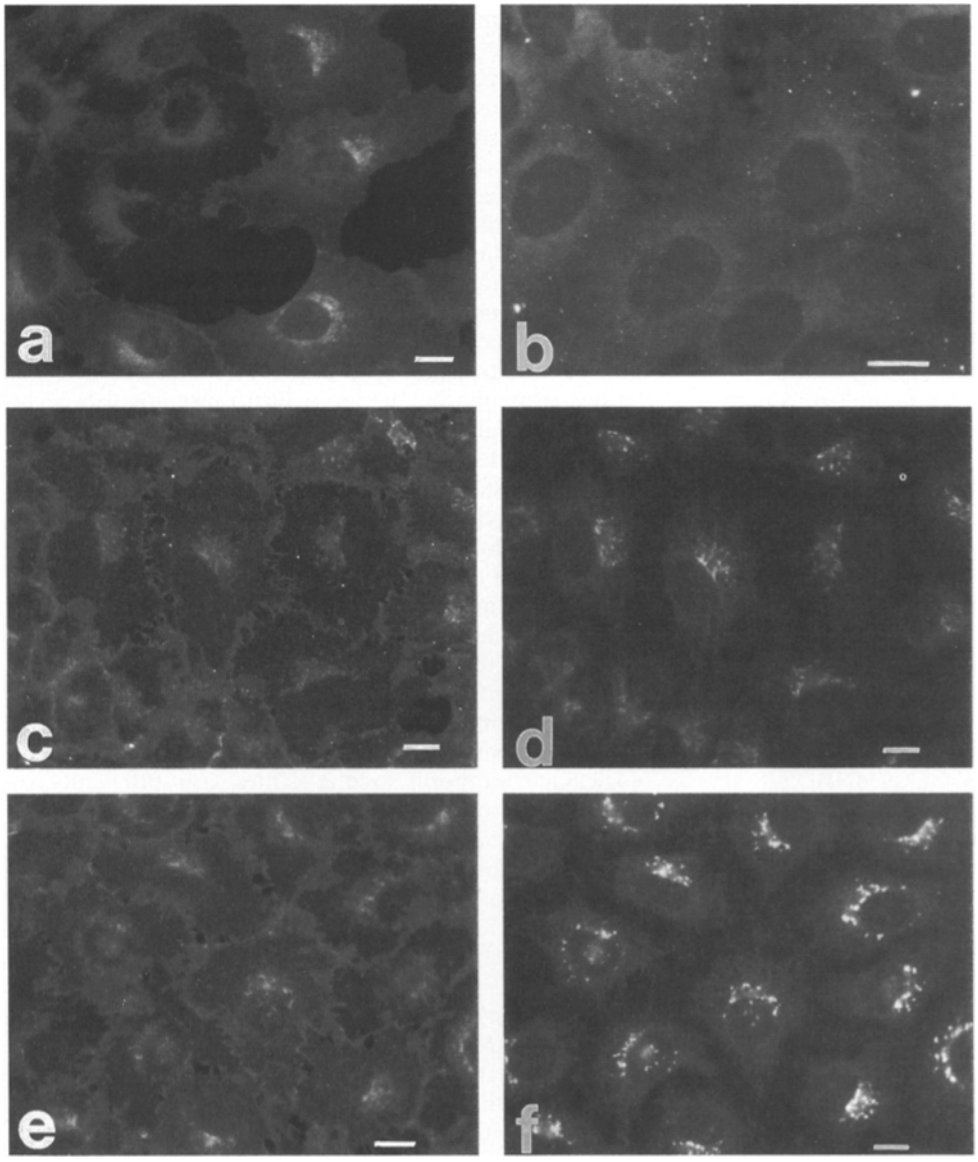

Figure 10. Fluorescence-microscopic localization of the intracellular site of S-acylation of myrC- and myrCG-edNBD in CV-1 cells. (*a*) Cells incubated with 50 μM myrC-edNBD for 2 h at 37°C. (*b*) Cells incubated with 50 μM myrS-edNBD for 4 h at 15°C (compare with cells similarly incubated with myrC-edNBD in (*e*). (*c* and *d*) Cells coincubated with 10 μM myrCG-edNBD and 40 μM C_8-DECA-ceramide for 4 h at 15°C: (*c*) NBD fluorescence; (*d*) C_8-DECA-ceramide fluorescence. (*e* and *f*) as (*c* and *d*), but using myrC-edNBD in place of myrCG-edNBD. Before microscopic observation all cell samples except that in (*b*) were washed with albumin as described in Materials and Methods; albumin washing of cells incubated as in (*b*) removed essentially all detectable fluorescence. Bar, 10 μm.

rated chromatographically from unmodified lipopeptide and quantitated by fluorescence to determine the absolute quantities of modified lipopeptide synthesized. Third, it is possible to employ unnaturally lipid-modified lipopeptides, representing potential S-acylation motifs that cannot be introduced into recombinant proteins (e.g., sequences containing terminal N-myristoylated amino acid residues other than glycine), to aid in mapping the specificity of the S-acylation process. Finally, the nondiffusible character of the lipopeptides in their S-acylated form (76) permits their selective localization in cells by fluorescence microscopy after removing unmodified lipopeptide with albumin. It is thereby possible to visualize the cellular membrane compartment(s) in which the S-acylation process takes place, particularly when cells are incubated with lipopeptides at reduced temperatures (4 or 15°C) where S-acylation still occurs but vesicle-mediated transport of materials between many membrane compartments is suppressed.

Various lines of evidence lead to the conclusion that the S-acylation of lipopeptides like those examined here by CV-1 and other mammalian cells represents an enzymatic activity. As already discussed in the Results section, comparison of the S-acylation of cellular proteins and of exogenous cysteinyl lipopeptides strongly suggests that the lipopeptide-acylation process does not primarily reflect the nonspecific abstraction of S-acyl groups from proteins by lipopeptides. The lipopeptide S-acylation process is equally unlikely to be mediated by nonenzymic transfer of S-acyl groups from cytoplasmic acyl donor molecules, for three reasons. First, cytoplasmic long chain acyl-CoAs, the most likely candidate donor for such a reaction, are normally tightly bound to specific intracellular binding proteins (59, 60) and in this form might not be expected to participate efficiently in nonspecific acyl transfer reactions. Second, the essentially absolute requirement of the S-acylation reaction for a lipophilic anchoring group on the peptide acceptor suggests that this reaction takes place within a membrane and not a cytoplasmic environment. Finally, the highly compartment-specific localization of the S-acylated products derived from the cysteinyl lipopeptides

studied here appears difficult to reconcile with the suggestion that S-acylation occurs by nonspecific acyl transfer from a cytoplasmic acyl donor.

Several points suggest that the plasma membrane–localized enzyme(s) mediating the S-acylation of the lipopeptides examined here may be the same as that responsible for the S-acylation of at least some plasma membrane-associated proteins. First, the lipopeptide acylating activity shows strong specificity for cysteinyl over serinyl residues and for membrane-associating over purely hydrophilic [lipo]peptides, although various types of membrane anchors can support S-acylation. These properties are very similar to those reported for the S-acylation of proteins such as G-protein α subunits and lck-homologous nonreceptor tyrosine kinases in cultured mammalian cells (2, 17, 25, 41, 52, 62, 64, 77, 78, 87, 90, 91). Second, the S-acylation of the representative lipopeptide myrGCG-edNBD resembles the S-acylation of cellular proteins in its sensitivity to inhibition by tunicamycin. Third, the finding that a variety of plasma membrane-associated proteins (including proteins bearing N-terminal myrGC- motifs) undergo dynamic S-acylation (16, 17, 39, 46, 51, 82, 86) strongly suggest that the plasma membrane itself may be an important locus of S-acylation (19), in agreement with our finding that this membrane is the preferred locus of S-acylation of lipopeptides bearing similar cysteine-containing motifs. Fourth, our finding that myrGCG-edNBD is S-acylated predominantly but not exclusively by palmitic acid in CV-1 cells is consistent with two recent reports (4,19) suggesting that palmitoyl-CoA is the preferred but not the exclusive acyl donor for in vitro S-acylation of certain lck-homologous tyrosine kinases and heterotrimeric G-protein α subunits. Finally, Berthiaume and Resh (4) have recently demonstrated an activity found in the membrane fraction of bovine brain lysate which mediates the palmitoyl-CoA–dependent S-acylation of recombinant myristoylated $p59^{fyn}$ and of chimeric constructs bearing an N-terminal myrGC- motif. The activity was found to be inhibited by myristoylated dodecapeptides bearing this motif, at concentrations similar to those at which the myrGC-based lipopeptides studied here serve as efficient substrates for S-acylation in intact cells. Unfortunately, the study just noted did not examine inhibition of protein S-acylation by shorter cysteinyl lipopeptides, nor did it determine whether (as our results would suggest) the myristoylated dodecapeptides serve as substrates as well as inhibitors for the S-acylation process.

Our findings that lipopeptides such as myrXCZ-edNBD are effective substrates for S-acylation at the plasma membrane but not the Golgi apparatus, while species such as myrCG-edNBD are readily S-acylated in both compartments, suggest that the two compartments contain distinct S-acylating activities with different intrinsic substrate specificities. The observation that lipopeptides bearing a myrGC- or -CXC(farn)-OMe motif are S-acylated (and consequently accumulated) preferentially at the plasma membrane is consistent with evidence suggesting that S-acylation of proteins bearing such motifs may target them specifically to this membrane compartment (25, 26, 78, 91). As we have noted previously (76), proteins bearing a myrGC- or -CXC(prenyl)-OMe motif that acquire a second (S-acyl) hydrophobic chain while associated with a particular membrane are expected to become kinetically trapped at the surface of that membrane until the S-acyl group is removed. The S-acylation of such motifs preferentially at the plasma membrane thus affords a simple mechanism for plasma membrane targeting (or sequestration) of proteins bearing these potential S-acylation sites. While lipopeptides such as myrC- and myrCG-edNBD represent obviously unphysiological S-acylation motifs, the membrane disposition of the cysteine residue in such lipopeptides (in contrast to that in the other lipopeptides examined here) may mimic that of cysteine residues lying precisely at the cytoplasmic junction between transmembrane and extramembrane sequences, as is the case for various proteins that undergo posttranslational S-acylation in a Golgi (or pre-Golgi transitional) compartment (56, 68). Further study will be required to determine within which subcompartment(s) of the Golgi complex the observed S-acylation of such lipopeptides takes place, and what other [lipo]peptide sequences and anchor groups may support S-acylation in this compartment.

The use of lipopeptides varying in their sequence and in their lipid anchor provides interesting information on the selectivity of the S-acylation process at the level of the plasma membrane. It is clear that the S-acylating activity (or activities) in this membrane can accept as substrates lipopeptides bearing a variety of physiological and unphysiological anchor motifs so long as these are sufficiently hydrophobic to promote membrane association. The very similar efficiencies of S-acylation observed for myristoylated and farnesylated lipopeptides with the core sequence -GCG- (Fig. 8 *D*) suggests that prenylated and N-myristoylated lipopeptides may be substrates for a common S-acylating activity. Our results with myristoylated lipopeptides of varying sequence also indicate that the glycine residue in the physiological myrGC- S-acylation motif, while optimal, is not essential for S-acylation. Instead, the efficiency of acylation appears to decrease, but is not abolished, as the sites flanking the modifiable cysteine are substituted with progressively bulkier (and particularly beta-branched) amino acid residues. This finding is consistent with observations that certain G_α proteins can be S-acylated when their normal NH_2-terminal myrGC- motif is replaced with an unmyristoylated AC- motif, and that -XC- motifs (where X may be a nonglycine amino acid) are S-acylated in wild-type G_α proteins such as α_s and α_{12}/α_{13}, if membrane anchorage is mediated by association of the α subunit with $G_{\beta\gamma}$ (16, 17, 35, 85, 90).

It remains to be determined whether one or multiple enzymes mediate S-acylation of plasma membrane-associated proteins, which vary both in the nature of their S-acylation sites and in their mode of membrane association. It is logical to suggest that the cysteinyl-containing lipopeptides examined here are substrates for the S-acyltransferase(s) that mediate(s) S-acylation of cellular proteins bearing similar cysteine-containing, lipid-modified terminal motifs. It is an open question whether the same S-acyltransferase(s) can mediate physiological S-acylation of integral membrane proteins, either on cysteine residues within or contiguous to transmembrane segments or on cysteine residues further removed from a transmembrane domain. Integral membrane proteins may of course present sites for S-acylation that differ markedly in sequence and/

or conformation from those found in reversibly membrane-associating proteins bearing myrGC- or -CX_nC(prenyl)-OMe motifs. It is however interesting to note that the plasma membrane–associated S-acyltransferase(s) acting on lipid-modified peptides exhibit(s) relatively broad specificity for both the cysteinyl-containing sequence to be modified and the means by which the cysteinyl residue is brought into proximity with the membrane surface. It is thus conceivable that at least some integral membrane proteins may serve as substrates for the plasma membrane-associated S-acyltransferase(s) detected here using lipopeptide substrates. Lipid-modified peptides may provide a useful tool (as substrates and, potentially, as inhibitors) to elucidate further the nature and possible diversity of plasma protein S-acyltransferases in future studies using intact or solubilized plasma membrane preparations.

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