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Functional analysis of protein N-myristoylation: Metabolic labeling studies using three oxygen-substituted analogs of myristic acid and cultured mammalian cells provide evidence for protein-sequence-specific incorporation and analog-specific redistribution

(fatty acid analogs/protein targeting/ras)

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ABSTRACT **Covalent attachment of myristic acid (C14:0) to the NH_2-terminal glycine residue of a number of cellular, viral, and oncogene-encoded proteins is essential for full expression of their biological function. Substitution of oxygen for methylene groups in this fatty acid does not produce a significant change in chain length or stereochemistry but does result in a reduction in hydrophobicity. These heteroatom-containing analogs serve as alternative substrates for mammalian myristoyl-CoA:protein *N*-myristoyltransferase (EC 2.3.1.97) and offer the opportunity to explore structure/function relationships of myristate in N-myristoyl proteins. We have synthesized three tritiated analogs of myristate with oxygen substituted for methylene groups at C6, C11, and C13. Metabolic labeling studies were performed with these compounds and (*i*) a murine myocyte cell line (BC_3H1), (*ii*) a rat fibroblast cell that produces $p60^{v\text{-}src}$ (3Xsrc), or (*iii*) NIH 3T3 cells that have been engineered to express a fusion protein consisting of an 11-residue myristoylation signal from the Rasheed sarcoma virus (RaSV) gag protein linked to c-Ha-ras with a Cys → Ser-186 mutation. This latter mutation prevents isoprenylation and palmitoylation of ras. Two-dimensional gel electrophoresis of membrane and soluble fractions prepared from cell lysates revealed different patterns of incorporation of the analogs into cellular N-myristoyl proteins (i.e., protein-sequence-specific incorporation). In addition, proteins were identified that underwent redistribution from membrane to soluble fractions after incorporating one but not another analog (analog-specific redistribution). Comparable studies using the model RaSV–ras chimeric protein also demonstrated analog-specific differences in incorporation, varying from ≈25% of the total RaSV–ras chimeric protein with 5-octyloxypentanoate to >50% with 12-methoxydodecanoate. Modification by this latter compound was so extensive that the amount of membrane-associated N-myristoylated protein was decreased. Incorporation of each of the analogs caused a dramatic redistribution to the soluble fraction, comparable to that seen when myristoylation was completely blocked by mutating the protein's site of myristate attachment (glycine) to an alanine residue. The demonstration that these analogs differ in the extent to which they are incorporated and in their ability to cause redistribution of any single protein suggests that they may also have sufficient selectivity to be of potential therapeutic value.**

N-myristoylation is a cotranslational (1), covalent protein modification involving linkage of a tetradecanoyl (myristoyl) group by means of an amide bond to the NH_2-terminal glycine residue of a number of cellular, viral, or oncogene-encoded proteins (2). Myristic acid is a rare fatty acid (FA) in mammalian cells (3). Addition of this acyl chain to form N-myristoyl proteins is catalyzed by myristoyl-CoA:protein *N*-myristoyltransferase (NMT; EC 2.3.1.97). NMT is highly selective for myristoyl-CoA *in vitro* (4) and *in vivo* (5, 6). *In vitro* studies suggest that this selectivity arises at least in part from a cooperativity between the enzyme's acyl-CoA- and peptide-binding sites (5). NMT selects fatty acyl-CoA substrates primarily by chain length rather than by hydrophobicity. Acyl-CoAs of the wrong chain length will dramatically alter the affinity of the enzyme for its peptide substrates, thereby reducing acyl-peptide production (5).

The role played by the myristoyl group in the function of N-myristol proteins has been explored by using two general approaches: (*i*) site-directed mutagenesis of their Gly-1 residues and (*ii*) the use of fatty acid analogs that have altered physical/chemical properties (compared to myristate) yet, when converted to their CoA thioesters, are still substrates for NMT. Deletion of the Gly-1 residue of $p60^{v\text{-}src}$ prevents its subsequent N-myristoylation, disrupts its stable association with the plasma membrane, and dramatically reduces its ability to tranform cells, although the oncoprotein's tyrosine kinase activity is not affected (7). Analogous mutagenesis of the Moloney murine leukemia virus $Pr65^{gag}$ (8), the $Pr78^{gag}$ of the Mason–Pfizer monkey virus (9), or the $Pr55^{gag}$ of human immunodeficiency virus 1 (10, 11) affects protein targeting and blocks viral assembly.

Studies with a fatty acid analog containing an oxygen substituted for a methylene group have suggested that different cellular N-myristoyl proteins exhibit different dependencies on the hydrophobic characteristics of their acyl moieties for targeting to cellular membranes (5). 10-(Propoxy)-decanoic acid (11-oxamyristic acid) has a similar chain length to C14:0 but a hydrophobicity comparable to decanoic acid (C10:0). Metabolic labeling studies using 10-(propoxy) [13,14(n)-^{3}H]decanoic acid and BC_3H1 cells revealed that only a subset of cellular N-myristoyl proteins incorporated the analog. Furthermore, only a small number of analog-substituted proteins demonstrated redistribution from membrane to cytosolic fractions (5). The sequence-specific nature of this single analog's incorporation and its protein-specific effects on membrane association indicated that heteroatom-containing FAs could be useful for exploring the physical/chemical features of myristate that are required for full expression of the biologic function of a N-myristoyl protein.

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Abbreviations: FA, fatty acid; NMT, myristoyl-CoA:protein *N*-myristoyltransferase; RaSV, Rasheed sarcoma virus; C/M, cytosolic to membrane.

Subsequent studies examining the effects of several different unlabeled oxygen- or sulfur-containing analogs on human immuodeficiency virus 1 assembly in H9 cells and Moloney murine leukemia viral replication in LZ1 cells (11) revealed that some of these compounds inhibited virus production without accompanying cellular toxicity and that different analogs had different effects on the two viruses. Although the precise mechanism leading to these effects was not defined, the results could have reflected differences in the efficiency of transfer of the various analogs to N-myristoyl protein(s) by cellular NMT and/or differences in the effects of analogs on protein function. Using three tritium-labeled analogs of myristate, we now show that the position of the heteroatom substitution can affect the efficiency of incorporation into, and can have dramatic effects on, the targeting of a given N-myristoyl protein.

MATERIALS AND METHODS

Synthesis of Radiolabeled Analogs of Myristic Acid with Reduced Hydrophobicity. Preparation of 10-(propoxy)-[13,14(n)-^{3}H]decanoic acid (11-oxamyristic acid) was described in an earlier publication (5). Tritiated 12-methoxydodecanoic acid (13-oxamyristic acid) and 5-octyloxypentanoic acid (6-oxamyristic acid) were prepared from the acetylenic acids $CH_3OCH_2C{\equiv}(CH_2)_8COOH$ and $CH_3(CH_2)_3C{\equiv}C(CH_2)_2O(CH_2)_4COOH$, respectively. The precursor to tritiated 13-oxamyristic acid was obtained by reacting methyl propargyl ether and iodononanoic acid in the presence of butyllithium. The product was purified by flash chromatography. The 6-oxamyristic acid precursor was prepared by first coupling 3-octyn-1-ol with *t*-butyl-5-bromovalerate in the presence of a phase transfer catalyst and then cleaving the *t*-butyl group with acid. The precursors [12-methoxy-10-dodecynoic acid and 5-(3-octynyloxy)pentanoic acid] were subjected to catalytic reduction with tritium gas to give 10,11- and 9,10-labeled 13-oxamyristic and 6-oxamyristic acids, respectively. The radiochemical purity of each compound was 99%. Their chemical identities were confirmed by mass spectrometry.

Metabolic Labeling Studies Using BC_3H1 Cells. T-75 flasks containing confluent monolayers of 3Xsrc (5) or differentiated BC_3H1 (5) cells and 5 ml of Dulbecco's minimal essential medium (DMEM), 10% delipidated (5) fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and pyruvate (5 mM) were incubated with 13-oxa[^{3}H]myristic acid (137 Ci/mmol, 1 mCi/ml of medium; 1 Ci = 37 GBq), 6-oxa[^{3}H]myristic acid (105 Ci/mmol, 1 mCi/ml), [9,10(n)-^{3}H]myristic acid (39 Ci/mmol, 0.2 mCi/ml), [9,10(n)-^{3}H]palmitic acid (54 Ci/mmol, 0.2 mCi/ml), or L-[^{35}S]methionine (800 Ci/mmol, 2 μCi/ml) at 37°C. At the end of the 4-hr labeling period, cells were washed twice with phosphate-buffered saline at 4°C, scraped into phosphate-buffered saline, pelleted by centrifugation at 250 × *g* for 10 min, and resuspended in 0.6 ml of a solution containing 100 mM KCl, 50 mM Tris (pH 7.5), leupeptin (5 μg/ml), and aprotinin (5 μg/ml). Cells were then broken at 4°C with 50 strokes of a Dounce homogenizer, and nuclei were removed by centrifugation for 10 min at 1000 × *g* (5). The resulting supernatant was centrifuged at 45,000 rpm for 30 min at 4°C in a type 50 Ti rotor (170,000 × g_{ave}). The protein concentration of the pellet ("membrane") and supernatant ("cytosol") fractions was determined by using the method of Bradford (12).

Two-dimensional gel electrophoresis of the radiolabeled membrane and soluble fractions was performed essentially as described by O'Farrell (13). Image analyses were performed with a BioImage Visage 2000 system (BioImage, Ann Arbor, MI). All quantitative measurements were performed on "spots" that were within the linear portion of the calibration curve for absorbance.

Metabolic Labeling Studies of NIH 3T3 Cells That Express the Chimeric gag–ras Protein p22-WT-Ser^{186} or its Nonmyristoylated Mutant p22(Ala^2)-WT-Ser^{186}. The DNA construct encoding p22-WT-Ser^{186} has been described (14). This protein consists of an 11-residue NH_2-terminal domain from the Rasheed sarcoma virus (RaSV) gag protein (Met-Gly-Gln-Ser-Leu-Thr-Lys-Gly-Gly-Thr-Ile) linked to the 189-amino acid nonactivated, or wild type (WT), human cellular Ha-ras (p21). p22(Ala^2)-WT-Ser^{186} was obtained by site-directed mutagenesis of Gly-2 of the RaSV gag sequence in p22-WT-Ser^{186}. Coding sequences of both DNAs (contained in a 1.6-kilobase *Bgl* II–*Bam*HI fragment) were inserted into the *Bam*HI site of the pZIP-NeoSV(x)1 vector (14). The DNAs were introduced into mouse NIH 3T3 cells (14). Stable transformants were selected based on their resistance to G418 and subsequently plated into 60-mm dishes. Cells were labeled with a mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine or ^{3}H-labeled FAs at confluency. For ^{35}S labeling, 1 ml of DMEM (cysteine and methione free) containing delipidated fetal calf serum (final concentration, 10%) was added to the dish together with 50 μM (unlabeled) FA analog and 100 μCi of the ^{35}S-labeled amino acids (Tran^{35}S-label; ICN). Alternatively, 2 mCi of the ^{3}H-labeled fatty acid (15–50 μM) was added per ml of DMEM/10% delipidated fetal calf serum. Following a 10- to 18-hr incubation, cells were subjected to hypotonic lysis followed by mechanical disruption with a Dounce homogenizer (14). Membrane and soluble fractions were prepared by centrifugation at 100,000 × *g* for 30 min (14). Ha-ras-containing proteins were recovered from each fraction by immunoprecipitation with a monoclonal antibody (146-3E4) that is specific for residues 157–181. (Note that most of the endogenous p21^{ras} in NIH 3T3 cells is Ki-ras, which is not recognized by this antibody.) Antigen–antibody complexes were isolated using a goat anti-mouse immunoglobulin (Cappel Laboratories) and Pansorbin (Calbiochem).

RESULTS AND DISCUSSION

Different Oxygen-Containing Analogs of Myristic Acid Are Selectively Incorporated into Distinct Subsets of N-myristoyl Proteins Synthesized in BC_3H1 and 3Xsrc Cells. Fig. 1 presents the results of a series of experiments designed to compare the patterns of incorporation of different FA analogs into cellular N-myristoyl proteins produced in two mammalian cell lines and their effects on protein targeting. Two tritiated analogs of comparable specific activity were used: one with an oxygen for methylene substitution near the ω end of the FA—12-methoxydodecanoic acid (13-oxamyristic acid)—and another with the substitution in the middle of the hydrocarbon chain—5-octyloxypentanoic acid (6-oxamyristic acid). These FA analogs are expected to be about 1% shorter than myristic acid and to maintain similar stereochemical properties (9). However, the 2-octanol/water partition coefficients indicate that these heteroatom-containing analogs have hydrophobicities comparable to that of a C10:0 FA (4). After a 4-hr incubation with either [^{3}H]myristic acid or one of the two analogs, cells were lysed, membrane and cytosolic fractions were prepared, and the radiolabeled proteins were separated by two-dimensional isoelectric focusing and SDS/PAGE. All of the membrane and soluble proteins visualized after incubating the murine myocyte-like cell line BC_3H1 with [^{3}H]myristate were also seen when 13-oxa[^{3}H]myristate was used (compare Fig. 1 *A* and *B* with *E* and *F*). By contrast, only a subset of cellular N-myristoyl proteins was labeled with 6-oxamyristate (Fig. 1 *C* and *D*). For example, proteins 1 and 2 were detected in both membrane and cytosolic (soluble) fractions when labeled with each of the three FAs. Proteins 3–6 were localized primarily in the soluble fraction after labeling with either [^{3}H]myristate or 13-oxa[^{3}H]myristate (Fig. 1 *B* and *F*). However, only proteins 4, 5, and 6 were detected in the cytosol after labeling with 6-oxa[^{3}H]myristate

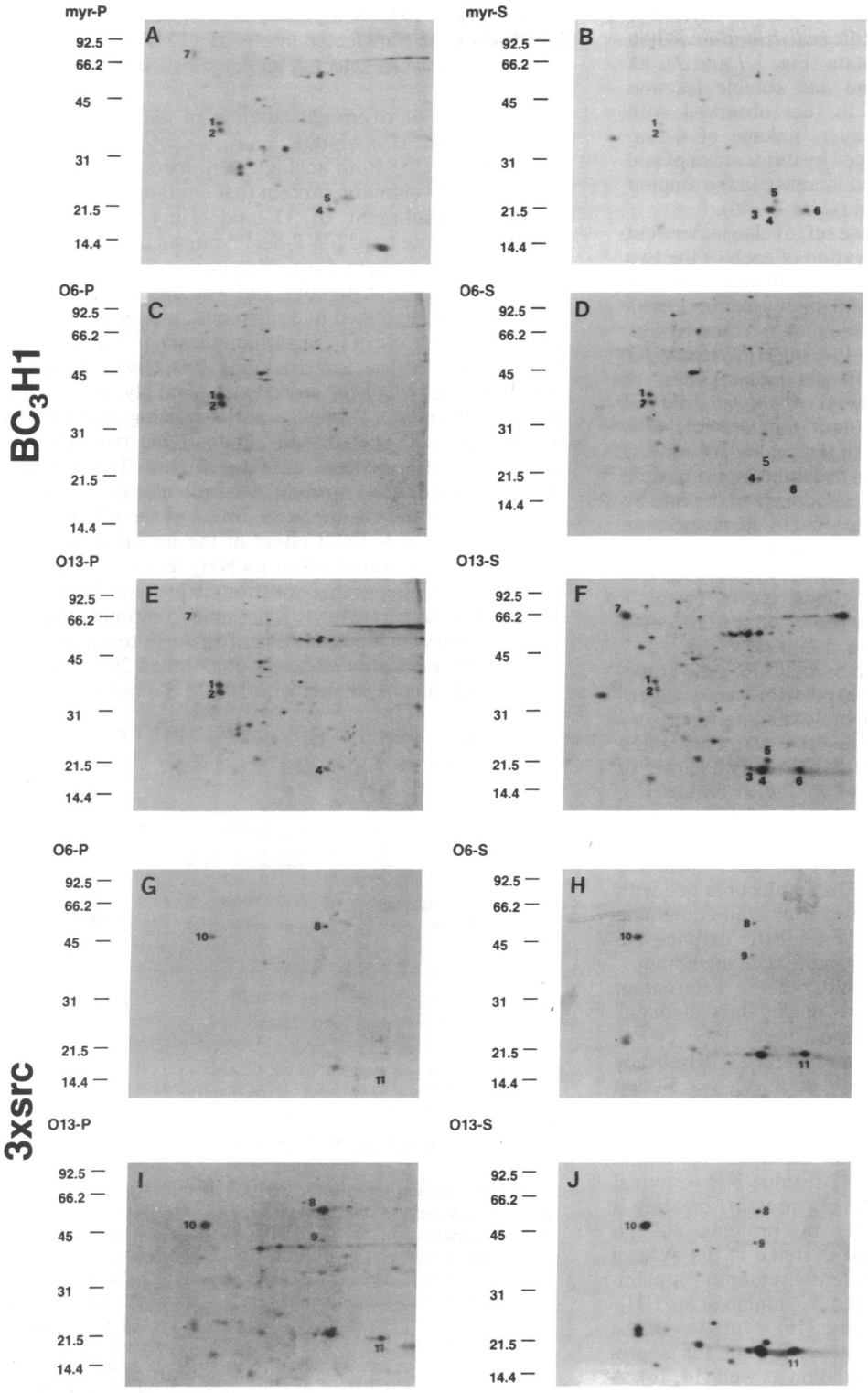

FIG. 1. Two-dimensional gel analyses of BC_3H1 (*A–F*) and 3Xsrc (*G–J*) cellular proteins labeled with [^{3}H]myristate (myr), 6-oxa[^{3}H]myristate (06), or 13-oxa[^{3}H]myristate (013). Fifty micrograms of total protein from the 170,000 × *g* membrane/cytosol pellet (P) or supernatant (S) fractions obtained from cell lysates was applied to each gel. The fluorographs were exposed for 60 days. Molecular size markers (in kDa) are given at left.

(Fig. 1*D*); none was noted in the membrane fraction (Fig. 1*C*). None of the proteins shown in Fig. 1 *A–F* was labeled with [^{3}H]palmitate (data not shown). This suggests that detection of ^{3}H label in a protein, after analog treatment, did not reflect elongation of the 14-carbon FA analog and its subsequent incorporation as an analog of palmitate (5). In addition, analog treatment did not affect the overall amounts of protein in membrane and cytosolic fractions as assessed by their Coomassie blue staining or [^{35}S]methionine labeling patterns (data not shown).

Analog incorporation caused a redistribution of only a subset of N-myristoyl proteins from the membrane to the cytosolic fraction. For example, proteins 1 and 2 are primarily membrane associated [cytosolic to membrane (C/M) ratio = 0.2 for both proteins] whether or not 6-oxamyristate or 13-oxymyristate is substituted for the C14:0 FA (Fig. 1 *A–F*). By contrast, when 13-oxamyristate was incorporated into protein 7, it underwent a dramatic redistribution (C/M ratio changed from 0.3 to 22; compare Fig. 1 *A* and *B* to *E* and *F*).

Evidence for Analog-Dependent and Specific Redistribution of Some N-myristoyl Proteins. Studies with 3Xsrc cells also disclosed analog-specific differences in *incorporation* (compare Fig. 1 *G–J*), suggesting that this phenomenon was not specific for BC_3H1 cells. Remarkably, careful inspection of

Fig. 1 *G–J* reveals several examples of cellular N-myristoyl proteins that underwent analog-specific *redistribution*. When protein 9 incorporated 13-oxamyristate (Fig. 1 *I* and *J*), its distribution between the membrane and soluble fraction (C/M ratio = 1.0) was identical to that observed with myristate (data not shown). However, linkage of 6-oxamyristate to the same protein (as judged by its identical pI and molecular weight) resulted in a 4-fold increase in the amount of labeled protein present in cytosol (C/M = 4.0).

$p60^{v\text{-}src}$ (protein 10 in Fig. 1 *G–J*, see ref. 5) also underwent marked redistribution upon incorporation of each of the two oxygen-substituted FA analogs: the C/M ratio for the N-myristoylated protein was 0.3 and increased to 1 with 13-oxamyristate and 3.5 after addition of 6-oxamyristate. When cells were pulse-labeled with 13-oxa[^{3}H]myristate for 2 hr and then incubated with a 10-fold molar excess of unlabeled myristate for 0, 4, or 10 hr, the C/M ratio did not change during the chase period (data not shown). This indicates that the cytosolic location of the analog-substituted $p60^{v\text{-}src}$ reflects a long-term change in the ability of the protein to interact with membranes and not a decrease in the rate by which the protein is eventually transported to the membrane.

Analog-specific effects on targeting are even more dramatically illustrated in 3Xsrc cells by protein 11. Incorporation of 13-oxamyristate had no significant effects on the protein's C/M ratio, whereas 6-oxamyristate produced a 6- to 7-fold increase in C/M to 50 (compare Fig. 1 *G–J*).

Previous metabolic labeling studies of COS cells transfected with cytomegalovirus-based expression vectors specifying six mammalian guanine nucleotide-binding regulatory protein (G protein) α subunits revealed that all incorporated [^{3}H]myristate and 11-oxa[^{3}H]myristate, but only a subset of the analog substituted proteins underwent redistribution (15). Thus, this single analog revealed that some of these homologous N-myristol proteins were apparently more dependent upon the hydrophobic properties of their acyl chain for membrane association than others. The results obtained with the BC_3H1 and 3Xsrc cells and several oxygen-containing analogs suggest a further way of operationally defining the interactions of a given N-myristoyl protein with membranes: acyl chains that differ in the position of the heteroatom substitution reveal analog-specific and analog-dependent differences in the targeting of the protein.

Analogs Can Be Used to Modify and Decrease Membrane Association of a Substantial Portion of a gag–ras Fusion Protein. We have identified a model protein for further examining and defining differences in analog incorporation and effects on targeting. It consists of the intact 189-amino acid human $p21^{ras}$ protein with an 11-residue NH_2-terminal extension derived from the RaSV gag protein, creating a 22-kDa protein, p22-WT-Ser^{186} (14). ras proteins undergo isoprenylation of a COOH-terminal cysteine in the cytosol (16–18), a modification that is apparently required for palmitoylation of another cysteine located 2–6 amino acids NH_2-terminal to the isoprenylated cysteine (16). Mutation of the COOH-terminal Cys-186 to Ser destroys the site for isoprenylation and thus prevents palmitoylation as well (14, 16). A mutant protein, p21-Leu^{61}-Ser^{186}, although activated by mutation of residue 61 to leucine, does not associate stably with the plasma membrane and cannot transform cells (14). However, linkage of the 11-residue gag myristoylation signal sequence allows the protein to be N-myristoylated and restores both its membrane targeting and transforming functions (14). Thus, our model protein p22-WT-Ser^{186} has no site for isoprenylation and subsequent palmitoylation and uses covalent attachment of myristate by means of an amide bond to the Gly-2 of the RaSV gag domain as the only means to target the protein to the membrane. A control chimeric protein was constructed that was identical to p22-WT-Ser^{186} except that a Gly-2 → Ala-2 substitution was introduced to block its ability to be N-myristoylated by cellular NMT. Both constructs were transfected into NIH 3T3 fibroblasts. Stable transformants were selected as described in *Materials and Methods*.

The results of overnight labeling of cells with [^{3}H]myristate, 11-oxa[^{3}H]myristate, and 13-oxa[^{3}H]myristate are shown in Fig. 2*A*. Both analogs were incorporated into the p22-WT-Ser^{186} chimeric protein that contains a Gly-2. Interestingly, attachment of 11-oxa[^{3}H]myristate and 13-oxa[^{3}H]myristate to p22-WT-Ser^{186} caused a reduction in the mobility of the protein during denaturing SDS/PAGE. This gel shift was quite distinctive and was observed even when the cells were incubated overnight with unlabeled analog (50 μM) in the presence of [^{35}S]methionine and [^{35}S]cysteine (see bands marked with an asterisk in Fig. 2*B*). Given the absolute requirement of NMT for an NH_2-terminal glycine residue in protein substrates (2), it was not surprising that the Ala-2 mutant was not labeled with either of the two ^{3}H-labeled analogs or [^{3}H]myristrate (data not shown). The mobility of the ^{35}S-labeled Ala-2 protein was not altered by analogs, indicating that the change in migration of the p22-WT-Ser^{186} protein was also a direct effect of the incorporation of the heteroatom-substituted FA at its NH_2 terminus. The ability to distinguish the analog-substituted protein from protein modified by myristate made it possible to estimate the extent (stoichiometry) of incorporation of analog into a protein. The upper, analog-substituted bands constituted 29% of the total p22 protein in cells treated with 50 μM 6-oxamyristate, 42%

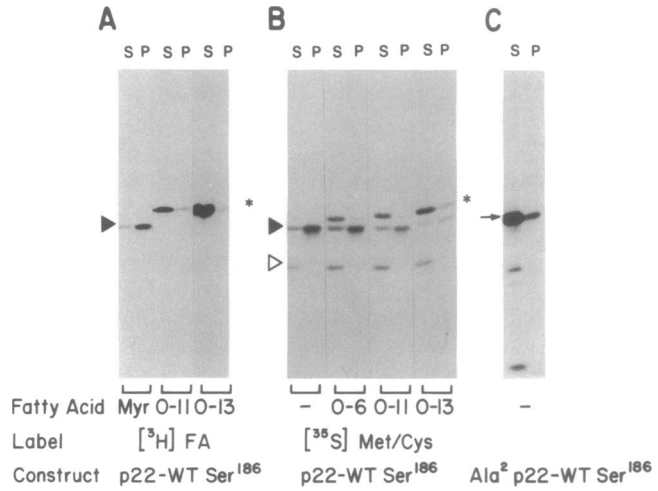

FIG. 2 Analog-specific effects on the targeting of a chimeric RaSV–ras protein (p22-WT-Ser^{186}) in mouse NIH 3T3 cells. (*A*) Cells were labeled overnight with [^{3}H]myristate or a ^{3}H-labeled analog, lysed, and subjected to centrifugation at 100,000 × *g*; immunoreactive proteins were recovered from the resulting pellet (P) and soluble (S) fractions with a monoclonal Ha-ras antibody. The fluorograph was exposed for 43 days {[^{3}H]myristate (Myr)} or 15 days {11-oxa[^{3}H]myristate (0–11) and 13-oxa[^{3}H]myristate (0–13)}. (*B*) Cells were incubated overnight with [^{35}S]methionine and [^{35}S]cysteine in the absence of analog or in the presence of 50 μM unlabeled 6-oxa[^{3}H]myristate (0–6), 11-oxa[^{3}H]myristate (0–11), or 13-oxa[^{3}H]myristate (0–13). Pellet and soluble fractions were prepared and subjected to immunoprecipitation as above. The asterisks in *A* and *B* indicate the position of migration of *analog*-substituted p22-WT-Ser^{186}. The closed arrowhead denotes the N-myristoylated p22-WT-Ser^{186} species. The open arrowhead is pointing to a p21-WT-Ser^{186} species derived by alternative initiation of translation of p22-WT-Ser^{186} mRNA at the methionine codon of its Ha-ras Ser-186 domain. The fluorograph was exposed for 2 days. (*C*) ^{35}S-labeled species immunoprecipitated from cells transfected with a recombinant vector that directs the synthesis of p22-WT-Ser^{186} with a Gly-2 → Ala-2 substitution. This mutation prohibits N-myristoylation. The exposure time for the fluorograph shown was 19 days. The arrow shows the ^{35}S-labeled, nonmyristoylated fusion protein.

of the total after treatment with 11-oxamyristate, and 81% after 13-oxamyristate treatment. This demonstrates clearly the differences and specificity of analog incorporation into the p22-WT-Ser^{186} protein. The incorporation of 13-oxamyristate is notable as it is an example where a majority of a N-myristoyl protein appears to be modified by the analog.

The effect of the analogs on the membrane association of the p22-WT-Ser^{186} protein was studied in two ways: (*i*) by labeling the protein with a ^{3}H-labeled analog or (*ii*) by treating cells with nonradioactive analog and detecting the analog-modified protein, labeled with [^{35}S]methionine and [^{35}S]-cysteine, by its unique mobility on gels. After incubation of cells with [^{3}H]myristate, 11-oxa[^{3}H]myristate, or 13-oxa[^{3}H]myristate, the majority of the N-myristoylated protein was membrane associated (C/M ratio = 0.2), whereas >90% of the analog-labeled p22-WT-Ser^{186} was found in the soluble fraction (Fig. 2*A*). Redistribution was also observed when cells expressing p22-WT-Ser^{186} were incubated overnight with 50 μM *unlabeled* analog and a mixture of [^{35}S]methionine and [^{35}S]cysteine. By comparing the relative intensities of the ^{35}S-labeled, shifted bands (noted by an asterisk in Fig. 2) in the pellet and supernatant fractions, we found that 6-oxa-, 11-oxa- and 13-oxamyristate produced comparable redistribution of p22-WT-Ser^{186} from membrane to soluble fractions. The C/M redistribution produced by incorporation of the analogs was equivalent to that seen with the nonmyristoylated Ala-2 mutant (Fig. 2*C*), which is primarily located in the cytosolic fraction (C/M ratio = 9). The extent to which 13-oxamyristate was incorporated was so great that the amount of myristoylated p22-WT-Ser^{186} in the membrane fraction was actually decreased.

An important internal control is provided by the ^{35}S-labeled species indicated by the open arrowhead (Fig. 2*B*). This most rapidly migrating species, immunoprecipitated by the anti-ras monoclonal antibody 146-3E4, represents p21-WT-Ser^{186}, a protein produced by alternative initiation of translation of the p22-WT-Ser^{186} mRNA at the initiator methionine of Ha-ras (retained in the chimeric protein) and not at the Met-1 of the 11-residue domain derived from RaSV. This nonactivated p21-WT-Ser^{186} (which is not acylated with either a myristoyl, farnesoyl, or palmitoyl group) is almost exclusively located in the cytosolic fraction, even when cells are incubated with analog, and demonstrates that the cell fractionation procedure was reproducible.

The efficiency of analog incorporation revealed by the data presented in Fig. 2*B* suggests that (*i*) these compounds are relatively stable in the cell culture system, (*ii*) they are transported across mammalian cell membranes, and (*iii*) they are substrates for both mouse CoA ligase(s) and NMT.

The redistribution produced by treatment with the three heteroatom-containing analogs at 50 μM was not sufficient to affect the morphology, or growth characteristics, of NIH 3T3 cells expressing the p22-WT-Ser^{186} protein. This may reflect the fact that $\approx$50% of the p22-WT-Ser^{186} species still contained a myristoyl group at its NH_2 terminus, rather than an oxamyristoyl moiety.

We do not yet know why different analogs have different effects on the distribution of this model protein. This, in part, reflects our ignorance about the environment that the myristoyl group "experiences" in the cell membrane, its conformation, the conformation of "its" protein, and what effect the acyl chain has on protein conformation. The altered interactions of some analog-substituted N-myristoyl proteins with membranes [e.g., p60$^{v\text{-}src}$ (protein 10) in 3Xsrc cells] may reflect perturbations in their interactions with specific receptors. Such a receptor has been identified for p60$^{v\text{-}src}$ (19). The analog data allow us to suggest that chain length is not the critical physical/chemical feature of the myristoyl group that contributes to targeting of p60$^{v\text{-}src}$ or the p22-WT-Ser^{186} model protein. Reductions in hydrophobicity equivalent to loss of approximately four methylene groups produced, in the case of p22-WT-Ser^{186}, almost complete redistribution (i.e., the solubility of the analog-substituted protein was equivalent to that of the nonmyristoylated Ala-2 protein). Although the three analogs used here did not vary greatly in their ability to cause this redistribution, 13-oxamyristate, because of its much greater incorporation, produced a larger amount of soluble p22. Thus, differences in incorporation can contribute to the specificity of analog effects. The results obtained in these studies emphasize the need to more closely characterize, compare, and contrast the interactions of N-myristoylated and analog-substituted proteins with model and biological membranes. We have recently developed a dual plasmid expression system that allows us to recapitulate this eukaryotic protein modification in *Escherichia coli* (6). Such a system may provide a useful means for generating nonmyristoylated, N-myristoylated, and analog-substituted forms of a given protein for such a comparative study and thereby provide insights about what physical/chemical features of myristate are critical for expressing its full biological function(s).

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