methods

Rapid and selective detection of fatty acylated proteins using ω-alkynyl-fatty acids and click chemistry[S]

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Abstract Progress in understanding the biology of protein fatty acylation has been impeded by the lack of rapid direct detection and identification methods. We first report that a synthetic ω-alkynyl-palmitate analog can be readily and specifically incorporated into GAPDH or mitochondrial 3-hydroxyl-3-methylglutaryl-CoA synthase in vitro and reacted with an azido-biotin probe or the fluorogenic probe 3-azido-7-hydroxycoumarin using click chemistry for rapid detection by Western blotting or flat bed fluorescence scanning. The acylated cysteine residues were confirmed by MS. Second, ω-alkynyl-palmitate is preferentially incorporated into transiently expressed H- or N-Ras proteins (but not non-palmitoylated K-Ras), compared with ω-alkynyl-myristate or ω-alkynyl-stearate, via an alkali sensitive thioester bond. Third, ω-alkynyl-myristate is specifically incorporated into endogenous co- and posttranslationally myristoylated proteins. The competitive inhibitors 2-bromopalmitate and 2-hydroxymyristate prevented incorporation of ω-alkynyl-palmitate and ω-alkynyl-myristate into palmitoylated and myristoylated proteins, respectively. Labeling cells with ω-alkynyl-palmitate does not affect membrane association of N-Ras. Furthermore, the palmitoylation of endogenous proteins including H- and N-Ras could be easily detected using ω-alkynyl-palmitate as label in cultured HeLa, Jurkat, and COS-7 cells, and, promisingly, in mice. The ω-alkynyl-myristate and -palmitate analogs used with click chemistry and azido-probes will be invaluable to study protein acylation in vitro, in cells, and in vivo.—Yap, M. C., M. A. Kostiuk, D. D. O. Martin, M. A. Perinpanayagam, P. G. Hak, A. Siddam, J. R. Majjigapu, G. Rajaiah, B. O. Keller, J. A. Prescher, P. Wu, C. R. Bertozzi, J. R. Falck, and L. G. Berthiaume. **Rapid and selective detection of fatty acylated proteins using ω-alkynyl-fatty acids and click chemistry. *J. Lipid Res.* 2010.** 51: **1566–1580.**

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For lipid synthesis, energy production via β-oxidation, or for protein fatty acylation to occur, long-chain fatty acids (LCFAs) must be activated by conversion to their CoA derivatives (LCFA-CoAs) by fatty acyl-CoA synthetase (FAS). Protein fatty acylation is one of many types of post-translational modifications of proteins by lipids, which also includes isoprenoids, glycosylphosphatidylinositols, and cholesterol. Typically, lipids covalently attached to proteins serve as hydrophobic membrane anchors (1–6).

Protein fatty acylation is mainly divided into two categories: N-myristoylation and S-acylation. The corresponding reactions are catalyzed by N-myristoyl transferases (NMT1 and NMT2) and two families of protein acyltransferases (PATs) referred to as zinc finger, Asp-His-His-Cys PATs

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Abbreviations: 2-BP, 2-bromopalmitate; EGFP, enhanced green fluorescent protein; ctPAK2, caspase-cleaved C-terminal p21 activated kinase 2; GFP, green fluorescent protein; HB, homogenization buffer; HMA, 2-hydroxy-myristic acid; HMGCS, 3-hydroxyl-3-methylglutaryl-CoA synthase; LCFAs, long-chain fatty acids; NA-HRP, NeutrAvdin™-HRP; NEM, N-ethylmaleimide; NMT, N-myristoyl transferase; PAT, protein acyltransferase; PVDF, polyvinylidene fluoride.

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and membrane bound O-acyl-transferases [reviewed in (5, 7, 8)]. In S-acylation, several LCFAs (e.g., C16:0, C16:1, C18:0, C18:1, and even C14:0) are found covalently attached to cysteine residues of proteins (9, 10). Palmitate is the most abundant fatty acid and, consequently, is preferentially attached onto proteins. As such, S-acylation is commonly referred to as palmitoylation. In N-myristoylation, the saturated 14 carbon fatty acid is added to an N-terminal glycine residue in proteins (11). A conservative G→A mutation is sufficient to block N-myristoylation, has been used extensively to abrogate myristoylation, assess its impact on protein function (11). There are two types of myristoylation based on the timing of the reaction, cotranslational and posttranslational. Cotranslational myristoylation occurs on glycine residues exposed by the action of a methionyl-aminopeptidase on nascent polypeptides, whereas posttranslational myristoylation occurs at cryptic internal glycine residues exposed following cleavage by caspases during apoptosis (11–18).

The long exposure time required to detect the incorporation of [^{3}H]fatty acids into protein (1–3 months or more) has long impeded the progress of investigators working on protein fatty acylation. The use of [^{125}I]iodo-fatty acids has reduced this significantly but is typically associated with handling of large quantities (mCi) of the hazardous isotope ^{125}I (19). Recently, we and others have used bio-orthogonal (analogs with functional handles that can be used by natural enzymes) ω-azido-fatty acid analogs to readily detect the acylation status of various fatty acylated proteins (16, 20, 21). Compared to using tritiated fatty acids in cell labeling reactions, the incorporation of the alkyl-azide analogs of fatty acids, ω-azido-dodecanoate (as an isosteric myristate analog), and ω-azido-tetradecanoate (as an isosteric palmitate analog) into proteins and their detection with a biotinylated-triarylphophine via the Staudinger reaction provided up to a million-fold increase in detection sensitivity (16). Using the palmitoyl-CoA analog ω-azido-tetradecanoyl-CoA as label, we identified 21 palmitoylated proteins in rat liver mitochondria, including 3-hydroxyl-3-methylglutaryl-CoA synthase (HMGCS), the rate-limiting enzyme in ketogenesis (21). Palmitoylated mitochondrial proteins are surprisingly numerous and the characterization of the role of their acylation is still pending in the vast majority of cases (21–24). In two characterized cases, the acylation of methylmalonyl semialdehyde dehydrogenase and carbamoyl phosphate synthetase 1 was shown to occur on the active site cysteine residues, thereby inhibiting these catabolic enzymes (22, 23). In addition, Gross et al. (25) showed that the glycolytic metabolic enzyme GAPDH is also a palmitoylated protein. Altogether, the large number of these palmitoylated metabolic enzymes suggests an imminent and underappreciated role for protein palmitoylation in the regulation of metabolism.

Another relatively recent breakthrough in the identification of palmitoylated proteins is the use of the acyl-biotin exchange reaction. The acyl-biotin exchange reaction is an indirect labeling method for the detection and purification of palmitoylated proteins from complex protein extracts. It substitutes biotinyl moieties for protein palmitoyl-modifications via a sequence of three chemical treatment steps. Now biotin-tagged proteins can be affinity-purified by streptavidin-agarose and protein identifications made by proteomic MS (26–28).

There are many different types of fatty acids attached to proteins. Therefore, the need for different chemical reporters to assess protein fatty acylation is essential. The alkynyl moiety has been used as a tag to generate bio-orthogonal analogs that could readily be reacted with azide-tagged probes using the Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition, a type of reaction also known as "click chemistry" (29–33). To complement the ω-azido-fatty acid series of chemical reporters (16, 20, 21), we sought to investigate the potential of ω-alkynyl-fatty acids and click chemistry as a means to detect S-acylation and N-myristoylation. In addition to increasing the reaction rate, the copper (I) catalyst also sensitizes alkynes toward dipolar reagents such as azides in a specific manner (33). Therefore, in the absence of copper (I), the ω-alkynyl-fatty acids remain practically inert in biological systems. Because various LCFAs are known to be incorporated into cysteine residues of palmitoylated proteins, the introduction of a triple C-C bond between carbons 15 and 16 of palmitate to produce the reactive ω-alkynyl-palmitate should not interfere with the physiological properties of the fatty acid analog (34). The use of ω-alkynyl-fatty acids to detect acylation of proteins has also recently been used by others (34, 35) and applied to perform proteomics studies on palmitoylated proteins in cells (26).

To assess the selectivity of incorporation of the ω-alkynyl-fatty acid analogs at a given site into proteins, we first took advantage of the fact that acylation of mitochondrial proteins occurred spontaneously and only required fatty acyl-CoA and purified enzymes. Second, another subset of well-characterized palmitoylated proteins is the Ras small GTPase family. All human Ras proto-oncogenes undergo extensive posttranslational modifications, including isoprenylation and carboxymethylation. In addition, H- and N-Ras (but not K-Ras) are also palmitoylated (6, 36–38) (supplementary Table I). To further validate the use of click chemistry for labeling of fatty acylated proteins in cultured cells and in vivo in mice, we also characterized the incorporation of the isosteric palmitic acid analog hexadec-15-ynoic acid (referred to as ω-alkynyl-palmitate) into a variety of H- and N-Ras constructs. To illustrate the versatility of this technique, we also show that an isosteric analog of myristic acid tetradec-13-ynoic acid (referred to as ω-alkynyl-myristate) can be incorporated co- and post-translationally into a variety of proteins.

In addition, to add further proof-of-concept and validation to this methodology, we show new results that confirm that the sites acylated by ω-alkynyl-palmitate are the same as palmitate by MS and provide new results that illustrate the versatility of the new method in vitro, in cells, and, promisingly, in vivo. We also show that labeling cells with ω-alkynyl-palmitate analog does not affect the subcellular fractionation pattern of N-Ras, which is predominantly found in the membrane fractions.

MATERIALS AND METHODS

Antibodies and reagents

All green fluorescent protein (GFP) antibodies were provided by Eusera (www.eusera.com, Edmonton, AB, Canada) or Abcam (Cambridge, UK). The following antibodies were used in this study: rabbit anti-GFP serum (Eusera EU-1 or Abcam Ab290; 1:50,000 dilution), goat anti-caspase-cleaved C-terminal p21 activated kinase (ctPAK2) (C19) (sc-1519, Santa Cruz Biotechnology; 1:1,000 dilution), affinity purified goat anti-GFP for immunoprecipitation (1 µl EU-4 from Eusera or Ab5450 from Abcam per sample), mouse anti-Fas for induction of apoptosis (clone CH11, Millipore; 150 ng/ml), rabbit anti-ctPAK2 serum for immunoprecipitation [10 µl per sample, prepared in the Berthiaume laboratory and described previously (16)], NeutrAvdin™ HRP (NA-HRP) conjugates (Pierce Biotechnology; 1:50,000), and rabbit anti-N-Ras (sc-519, Santa Cruz Biotechnology; 1:500). Crude rat hybridoma supernatants containing anti-pan Ras 259 or rat anti-H and K-Ras 238 (20 µl per sample) originally from ATCC were kind gifts from Dr. Jim Stone (University of Alberta, Canada). Monoclonal mouse anti-Ras clone RAS10 was from Millipore (used at 1:2,000). Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available.

DNA constructs

The pEGFP-N1 vector was purchased from Invitrogen (Carlsbad, CA). The WT-ctPAK2-N_{15}-EGFP and G2A-ctPAK2-N_{15}-EGFP constructs were from previous work (15) based on the pEGFP-N1 vector. Wild-type, full-length human Ras constructs, EGFP-H-Ras, EGFP-N-Ras, and EGFP-K-Ras, were gifts of Dr. Patrick Casey (Duke University Medical Center, Durham, NC). These constructs were based on the pEGFP-C1 vector (Clontech) in which the enhanced GFP was fused to the N-terminus of H-Ras, N-Ras, or K-Ras4B.

Cell culture

COS-7 and Jurkat T cells were purchased from the ATCC (Manassas, VA). We obtained HeLa cells from Dr. T. Simmen (University of Alberta). All reagents for cell culture were purchased from Invitrogen. COS-7 and HeLa cells were maintained in DMEM while Jurkat cells were cultured in RPMI medium at 37°C and 5% CO_2 in a humidified incubator. All the maintenance media were supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Cell transfection

COS-7 cells were transiently transfected with the indicated constructs using FuGene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) per the manufacturer's instructions in DMEM supplemented with 10% FBS in the absence of antibiotics. Transfection reagent-DNA complex was left in the medium for 16 h.

Metabolic labeling of cells with ω-alkynyl- and ω-azido-fatty acids

The ω-alkynyl- and ω-azido-fatty acids synthesized as described in the supplementary material file (soon available from www.eusera.com) were added to cells as described in Martin et al. (16) with the following modifications. Cells were deprived of fatty acids by incubating in their respective media supplemented with either 5% dextran-coated charcoal-treated FBS or 1% fatty acid-free BSA (Sigma Aldrich) for 1 h prior to labeling. The ω-azido-fatty acids, ω-alkynyl-fatty acids, or fatty acids were dissolved in DMSO to generate 20–100 mM stock solutions. To facilitate cellular uptake of ω-azido-fatty acids or fatty acids, prior to labeling, these were saponified by incubation with a 20% molar excess of potassium hydroxide at 65°C for 15 min. To do so, a 20× solution was made by dissolving the saponified fatty acids in prewarmed, serum-free culture media containing 20% fatty acid-free BSA at 37°C, followed by an additional 15 min incubation at 37°C. After deprivation of fatty acids, cells were washed with warm PBS and incubated in fresh media without supplement. One-twentieth volume of the 20× fatty acid-BSA conjugate in serum-free media was added to the cells, typically 200 µl to 3.8 ml media, so that the final concentration of BSA was 1% and the fatty acids were at the indicated concentrations. Saponification of the ω-alkynyl-fatty acids was also performed but was not required to increase incorporation into proteins. In control experiments, mock DMSO or unlabeled fatty acids were subjected to the saponification and conjugation to BSA as described above. Cells were labeled for times indicated at 37°C in a 5% CO2 humidified incubator. The inhibitors of N-myristoylation and S-acylation, 2-hydroxy-myristate (HMA) and 2-bromopalmitate (2-BP), respectively, were also saponified and conjugated to BSA prior to addition to cells.

Immunoprecipitation

Cells ($\sim 1 \times 10^6$ to 1×10^7) were washed with cold PBS, harvested, and lysed with cold EDTA-free RIPA buffer [0.1% SDS, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium-deoxycholate, 2 mM $MgCl_2$, EDTA-free complete protease inhibitor (Roche)] by rocking for 15 min at 4°C. Cell lysates were centrifuged at 16,000 *g* for 10 min at 4°C and the postnuclear supernatants were collected. GFP fusion proteins were immunoprecipitated from approximately 1 mg of protein lysates with affinity purified goat anti-GFP cross-linked to Sepharose beads (www.eusera.com) by rocking for 2 h or overnight at 4°C. The beads were extensively washed with 0.1% SDS-RIPA, resuspended in 50 mM HEPES, pH 7.4, with 1% SDS, and heated for 15 min at 80°C. The supernatants containing the fusion proteins were collected. For the immunoprecipitation of endogenous Ras, rat anti-pan Ras 259 or rat anti-H and K-Ras 238 antibodies immobilized to protein G sepharose beads (GE Healthcare) were used as described above and immunoprecipitated from 4–5 mg of protein lysate. Endogenous PAK2 was immunoprecipitated from 300 µg of Jurkat protein lysate using rabbit anti-ctPAK2 serum.

Radiolabeling of cells and detection of radiolabeled proteins

Transfected COS-7 cells ($\sim 1 \times 10^6$ to 1×10^7) were deprived of fatty acids by incubating in serum free DMEM supplemented with 0.1% fatty acid-free BSA. [9,10(*n*)-^{3}H] Myristic acid and [9,10(*n*)-^{3}H] palmitic acid (500 µCi) were saponified by 20% molar excess of potassium hydroxide at 65°C for 15 min. Saponified fatty acids were dissolved and incubated in 0.1% fatty acid-free BSA in DMEM for 15 min at 37°C, followed by 5 min at 65°C, then returned to 37°C before adding to the cells. Cells were radiolabeled for 4 h at 37°C then lysed with 0.1% SDS-RIPA buffer. Radiolabeled proteins were immunoprecipitated as described above with goat anti-GFP Sepharose beads rocking overnight at 4°C. Immunocomplexes were separated by SDS-PAGE, blotted onto polyvinylidene fluoride (PVDF) membranes, and detected by rabbit anti-GFP serum. PVDF membranes containing radiolabeled proteins were air-dried and exposed to Biomax MS film for 50 days.

Subcellular fractionation

Metabolically labeled cells were fractionated as previously described (39, 40). Two confluent 10 cm plates of COS-7 cells were

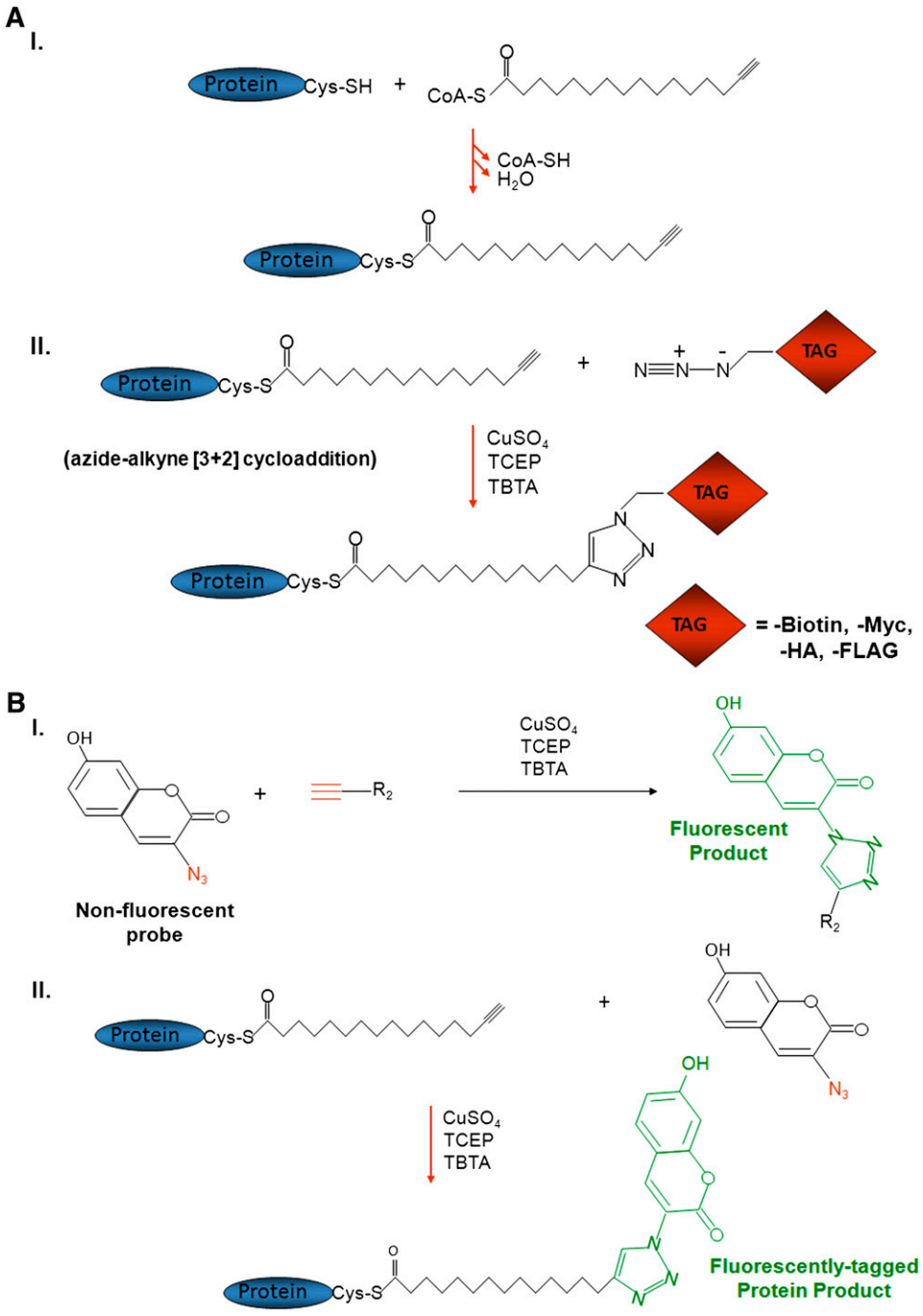

Fig. 1. Using ω-alkynyl-palmitate analog as a chemical reporter to label proteins in vitro and in vivo. Prior to their incorporation into proteins in vitro or in vivo, fatty acids were activated into their CoA ester (A). Proteins acylated with ω–alkynyl-palmitate analog are detected using copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition (click chemistry) with an azido-tag (e.g., azido-biotin) (A) or the fluorogenic probe 3-azido-7-hydroxycoumarin (B) (43).

washed twice with cold HEPES buffer (150 mM NaCl, 50 mM HEPES, pH 7.4), scraped off the plates using a cell lifter, and harvested. Cells were spun down, resuspended, and swelled in hypotonic buffer, Mg resuspension buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4 with EDTA-free complete protease inhibitors) for 20 min on ice, and homogenized with 30 strokes using the tight pestle in a Dounce homogenizer. Following the addition of 0.4 vols of EDTA-free 2.5× homogenization buffer (HB) (625 mM sucrose, 25 mM HEPES, pH 7.4 with EDTA-free complete protease inhibitors) to the homogenate, the cells were homogenized with 10 more strokes using the loose pestle. Ten percent of the homogenate (T) was saved and SDS was added to a final concentration of 1%. The rest (90%) of the homogenate (T) was centrifuged at 1,000 *g* for 5 min at 4°C. The postnuclear supernatant (S1) was collected. The pellet (P1), containing mostly nuclei, was washed once with 1× HB (250 mM sucrose, 10 mM HEPES, pH 7.4 with EDTA-free complete protease inhibitors) and homogenized in 1× HB containing 1% SDS with 10 loose strokes. Fractions T and P1 were passed through a 26 G hypodermic needle to shear the DNA. S1 was further centrifuged

at 15,000 *g* for 10 min at 4°C, yielding a heavy membrane pellet enriched in mitochondria and microsomes (P10). The supernatant was collected and centrifuged at 100,000 *g* for 30 min at 4°C in a Beckman TLA 120.2 rotor, resulting in cytosolic fraction (S100) and a light membrane pellet (P100). The P10 pellet was washed once with 1× HB. Both P10 and P100 pellets were solubilized in 1× HB with 1% SDS while the S100 fractions were adjusted to 1% SDS.

Mitochondrial isolation from rat primary hepatocytes

Primary cultures of rat hepatocytes were a kind gift of Dr. David N. Brindley, University of Alberta, Canada. Crude mitochondrial fractions were obtained using differential centrifugation as described in Corvi et al. (23). All buffers contained freshly added EDTA-free Complete protease inhibitor cocktail.

Labeling of proteins with ω-azido-palmitoyl-CoA and ω-alkynyl-palmitoyl-CoA in vitro

Recombinant mature HMGCS-His6 was purified as described in Kostiuk et al. (21). The synthesis of ω-alkynyl-palmitoyl-CoA from ω-alkynyl-palmitate was performed as described for that of ω-azido-palmitoyl-CoA from ω-azido-palmitate analog (21). Incubations were carried out in a final volume of 50–100 μl using 1–3 μg purified HMGCS-His6 or GAPDH proteins or 10 μg of crude liver mitochondria with 50 μM final azido-palmitoyl-CoA or ω-alkynyl-palmitoyl-CoA for 30 min at 25°C as described in Kostiuk et al. (21). At the end of the incubation period, reactions containing the ω-alkynyl-palmitoyl-CoA were adjusted to 1% SDS and reacted with the various azido-tags as described below. For the palmitoyl-CoA competition experiments, purified HMGCS-His6 or GAPDH were preincubated for 45 min in the presence of various concentrations of palmitoyl-CoA as detailed in the figure legend prior to labeling as above.

Detection of ω-azido-fatty acid labeled proteins by Staudinger ligation

The detection method was adapted from Martin et al. (16). Cell lysates or immunoprecipitated proteins (eluted from the beads as described above) were adjusted to 1% SDS and reacted with 250 μM of phosphine-biotin in the presence of 10 mM DTT at 37°C for 2 h in darkness. Reactions were terminated by the addition of 5× SDS-PAGE sample buffer (4% SDS, 25% glycerol, 0.1% bromophenol blue, 100 mM DTT, 60 mM Tris-HCl, pH 6.8). Samples were boiled for 5 min then separated by SDS-PAGE and blotted onto PVDF membranes. Detection of biotinylated-azido-fatty acylated proteins was performed by probing the membranes with NA-HRP (dilution: 1:25,000–50,000). The blots were developed with ECL Plus Western blotting reagent (GE Healthcare) and exposed to low sensitivity Kodak film. Figure results are representative of two or more experiments.

Detection of ω-alkynyl-fatty acid labeled proteins using click chemistry

Cell lysates or immunoprecipitated proteins (eluted from the beads as described above) were adjusted to 1% SDS and incubated with 100 μM Tris-(benzyltriazolylmethyl)amine, 1 mM $CuSO_4$, 1 mM Tris-carboxyethylphosphine, and 100 μM azido-biotin or 3-azido-7-hydroxycoumarin at 37°C in darkness for 30 min. Reactions containing the azido-biotin were stopped by the addition of 10 vol of ice-cold acetone and proteins were precipitated at −20°C for 1 h or overnight. Precipitated proteins were spun down at 16,000 *g* for 15 min and resuspended in 1× SDS-PAGE sample buffer containing 20 mM DTT. Reactions containing the 3-azido-7-hydroxycoumarin were stopped by addition of one-fifth volume 5× SDS-PAGE sample buffer. Samples were heated at 95°C for 5 min then analyzed by SDS-PAGE. Gels containing the products of the fluorogenic reactions with the 3-azido-7-hydroxycoumarin were scanned with a Storm 840 Phosphorimager in the Blue fluorescence mode. Membranes were probed as described above. Figure results are representative of two or more experiments.

Potassium hydroxide treatment of PVDF membranes

When PVDF membranes were treated with KOH, protein samples were loaded in duplicate on separate SDS-polyacrylamide gels. After electrophoresis and blotting, one of the duplicate membranes was soaked in 100 ml of 0.1 N KOH in methanol [1 N KOH in H_2O:methanol 1:9 (v/v)], and the other was soaked in 0.1 N Tris-HCl pH 7.0 in methanol made from 1 N Tris-HCl, pH 6.8:methanol 1:9 (v/v) at 25°C for 45 min with gentle shaking. Treated membranes were washed thoroughly with PBS before probing with NA-HRP/ECL.

In vivo labeling of Ras proteins with ω-alkynyl-palmitate in BALB/c mice

Assuming a 2 ml blood volume for BALB/C mice, we injected 200 μl of a 10× stock solution containing 1 mM ω-alkynyl-palmitate or palmitate conjugated to BSA prepared as described above (except the saponified fatty acids were dissolved in prewarmed PBS containing 2% fatty acid-free BSA) via the mouse tail vein. This resulted in an approximate 100 μM circulating concentration of ω-alkynyl-palmitate. After 1 or 4 h postinjection, livers were excised and a homogenate was made as described in Corvi et al. (23). Samples (5 mg) of postnuclear supernatant (1,000 *g*) were solubilized in RIPA buffer and used in immunoprecipitation experiments with rat anti-pan-Ras 259 antibody as described above. We are currently optimizing delivery and uptake kinetics of the ω-alkynyl-palmitate analog into mice. All animal handling was done as per our protocol approved by the University of Alberta Animal Welfare Committee. Of note, mice injected with ω-alkynyl-palmitate or palmitate conjugated to BSA behaved normally and did not show any sign of distress in our experimental protocols lasting up to 4 h. A mouse injected with ω-alkynyl-palmitate as described above showed no adverse effect for 6 months. The long-term effects of ω-alkynyl-palmitate injections into mice are currently being assessed.

RESULTS

Palmitoylation of enzymes in vitro is selective and specific

Two previously identified palmitoylated proteins, GAPDH (25) and recombinant HMGCS (21), were purified and subjected to in vitro labeling with ω-alkynyl-palmitoyl-CoA followed by addition of azido-biotin or 3-azido-7-hydroxycoumarin, via a copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition reaction (**Fig. 1**) and separation of the products by denaturing, reducing SDS-PAGE. The biotinylated proteins were detected by Western blotting with NA-HRP, and the azido-coumarin labeled proteins were detected by flat bed fluorescence scanning. As shown in **Fig. 2A, C**, the acylation of HMGCS and GAPDH was readily detected using NA-HRP/ECL when ω-alkynyl-palmitoyl-CoA was used as label, but not when the ω-alkynyl-fatty acid moiety was omitted from the reaction. Of note, the acylation reaction required the CoA-activated form of the ω-alkynyl-palmitate (data not shown). To determine whether the ω-alkynyl-palmitate was bound to cysteine

residue(s), we preincubated the enzymes with the cysteine alkylating reagent N-ethylmaleimide (NEM) prior to labeling with ω-alkynyl-palmitoyl-CoA and Cu^+-catalyzed reactions, as described above. Neither of the proteins incorporated the label when preincubated with NEM, strongly suggesting that the ω-alkynyl-palmitate is covalently attached to the enzymes at cysteine residue(s) (Fig. 2A, C, lane 3). To further determine whether the alkynyl-palmitate was bound to HMGCS and GAPDH at the same cysteine residues as the naturally occurring palmitate, we performed competition experiments. When the enzymes were preincubated with increasing amounts of palmitoyl-CoA prior to labeling with ω-alkynyl-palmitoyl-CoA and Cu^+-catalyzed azide-alkyne cycloaddition, we saw a palmitoyl-CoA concentration-dependent reduction in labeling intensities for both enzymes (Fig. 2, B, D). This data strongly suggests that the ω-alkynyl-palmitate is binding the same cysteine residue(s) as palmitate itself. When we attempted to investigate the direction of the click chemistry reaction by labeling HMGCS with an ω-azido-palmitoyl-CoA analog followed by copper (I)-catalyzed cycloaddition using an alkynyl-biotinylating compound, we obtained increased background levels (data not shown). Under our experimental conditions, the alkynyl-biotin appeared to react nonspecifically with numerous proteins in the presence of copper (I). This nonspecific phenomenon was also previously described by others (32, 41).

The identity of the HMGCS cysteine residue(s) acylated in the presence of 50 μM palmitoyl-CoA, ω-azido-tetradecanoyl-CoA, or ω-alkynyl-palmitoyl-CoA was confirmed using MS. In **Fig. 3**, we show that in all three analyses of the tryptic peptide containing Cys305, the observed m/z (m/z = 3,068.5, m/z = 3,064.5 and m/z = 3,081.5 for the palmitoylated, ω-alkynyl-palmitoylated, and ω-azido-tetradecanoylated peptide, respectively) are well in line with the calculated m/z. In the absence of incubation with LCFA-CoAs, the peptide containing Cys305 was readily observed at m/z = 2,830.3 but not when HMGCS was incubated with the above-mentioned acyl-CoA derivatives, suggesting that the acylation of Cys305 was complete under those conditions (supplementary Table II). These results combined with the competition assays in Fig. 2B, D confirmed that the ω-alkynyl-palmitate is being incorporated at the same cysteine residue as palmitate in HMGCS.

We previously showed that numerous mitochondrial proteins are palmitoylated (21–23). We then analyzed the ability of the ω-alkynyl-palmitate to be imported into the mitochondria and palmitoylate mitochondrial enzymes. We labeled mitochondria isolated from primary rat hepatocytes with ω-alkynyl-palmitate and the necessary cofactors required to import fatty acids into the mitochondria (22). After termination of the reactions with 1% SDS final concentration, the mitochondrial proteins were subjected to Cu^+-catalyzed azide-alkyne cycloaddition with azido-biotin. Biotinylated proteins were then visualized by Western blot and ECL using NA-HRP. The results in supplementary Fig. I show that multiple mitochondrial proteins are labeled only when ω-alkynyl-palmitate was present in the labeling reaction. The bands present at apparent molecular weights of 130 kDa and 75 kDa, present in all lanes, can be attributed to endogenously biotinylated proteins (likely carboxylases) being detected by the NA-HRP/ECL reaction.

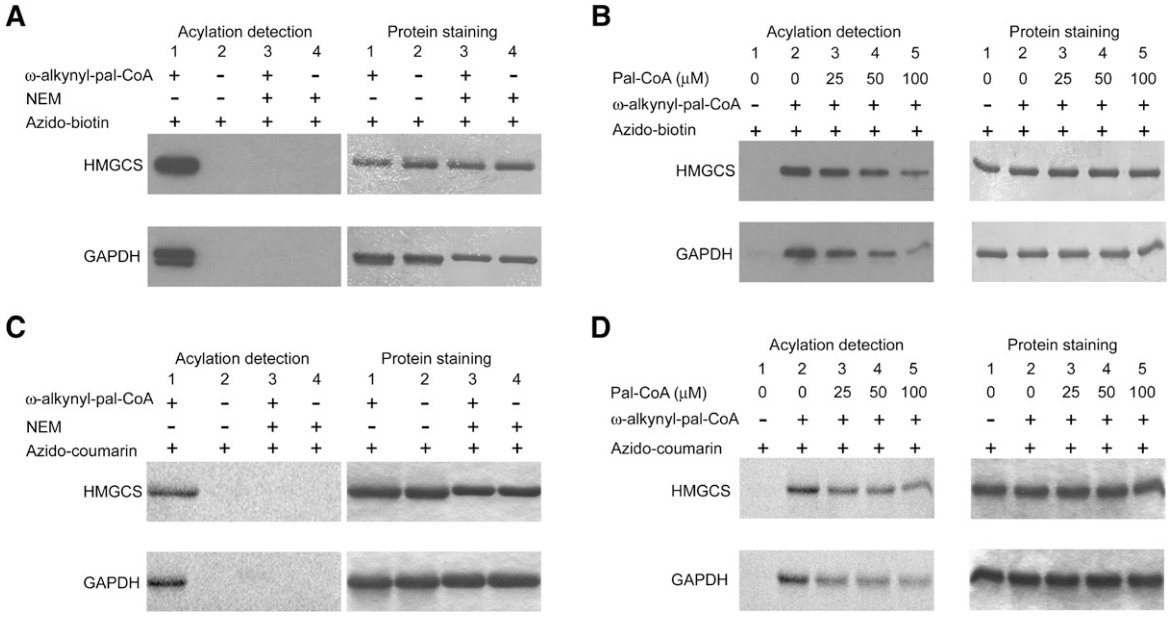

Fig. 2. Detection of ω-alkynyl-palmitoylated proteins labeled with azido-biotin or 3-azido-7-hydroxycoumarin using Cu^+-catalyzed alkyne-azide cycloaddition. A: A total of 500 ng of purified HMGCS or GAPDH was labeled using 50 μM ω-alkynyl-palmitoyl-CoA or CoA as a negative control as indicated followed by Cu^+-catalyzed cycloaddition with either azido-biotin (A and B) or 3-azido-7-hydroxycoumarin (azido-coumarin) (C and D). Reactions were separated by SDS-PAGE and reaction products detected on membranes by Western blotting with NA-HRP (A and B) or by direct fluorescence scanning of the polyacrylamide gel (C and D). Corresponding Coomassie blue staining of the membranes or gels is shown on the right (protein staining). Where indicated, enzymes were preincubated in the presence of 10 mM NEM for 30 min prior to additional incubations and Cu^+-catalyzed cycloadditions.

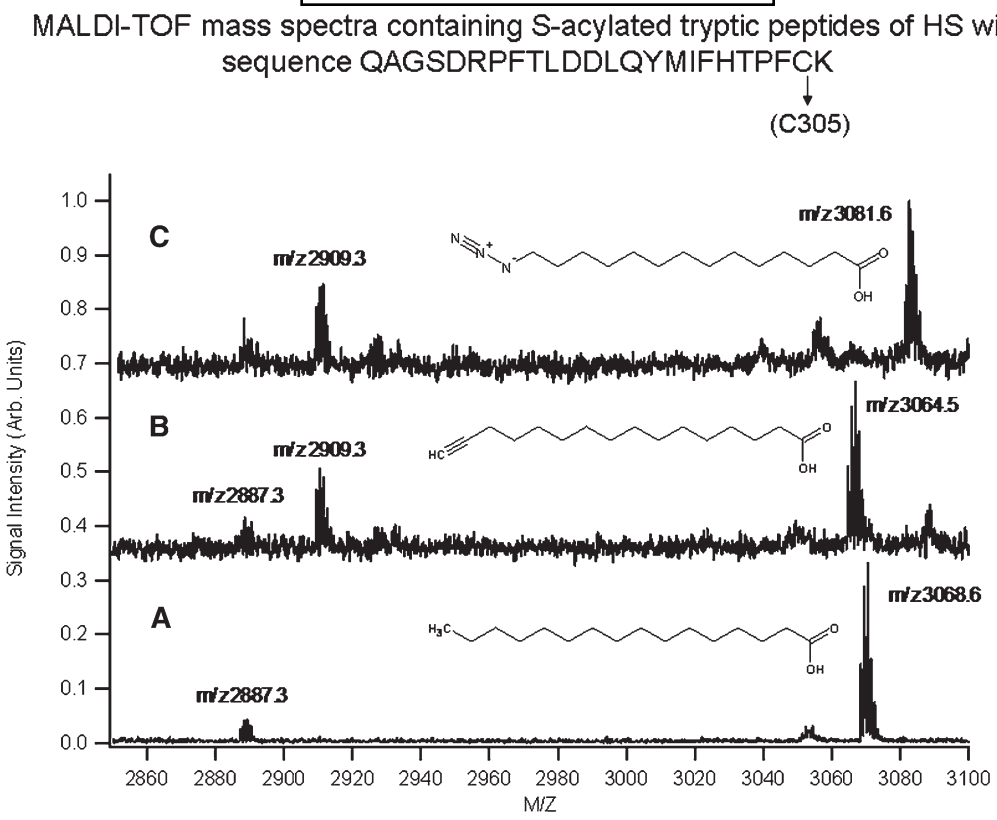

Fig. 3. MALDI time-of-flight mass spectra excerpts of tryptic digests of HMCGS-His6 S-acylated with different palmitate analogs. Following incubation of HMGCS-His_6 with palmitoyl-CoA (A), ω-alkynyl-palmitoyl-CoA (B), or ω-azido-tetradecanoyl-CoA (C) alkylation, trypsinization, and reduction, the spectra demonstrate the complete acylation of the peptide containing Cys305 (QAGSDRPFTLDDLQYMIFHTPFC305 K). Observed *m/z* values at 3,068.6, 3,064.5, and 3,081.6 are for the acylated peptide containing attached (A) palmitate, (B) alkynyl-palmitate, and (C) azido-palmitate moieties, respectively. The signals at *m/z* 2,887.3 ($[M+H]^+$) and *m/z* 2,909.3 ($[M+Na]^+$) are from another nonpalmitoylated peptide with sequence GTHMENVYDFYKPNLASEYPIVDGK.

The incorporation of ω-alkynyl-myristate and -palmitate into proteins is selective and specific

Because H-, N-, or K-Ras isoforms vary in sequences only in their C-terminal regions (supplementary Table I), they offer a unique opportunity to be used as tools to show proof-of-principle and assess the labeling specificity provided by various fatty acids or their analogs. To establish whether the ω-alkynyl-palmitate analog could be incorporated into proteins in cultured cells, we metabolically labeled COS-7 cells transiently transfected with plasmids expressing EGFP-tagged H-, N-, or K-Ras with ω-alkynyl-palmitate (100 μM) as we did with ω-azido-myristate analog in Martin et al. (16) and described in Materials and Methods. Following solubilization in RIPA buffer and immunoprecipitation with goat anti-GFP antibodies, the immunoprecipitated EGFP-tagged proteins were subjected to Cu^+-catalyzed azide-alkyne cycloaddition, leading to biotinylation of the ω-alkynyl-fatty acylated proteins. In **Fig. 4A**, we show that EGFP-tagged H- and N-Ras isoforms (but not EGFP-K-Ras or EGFP) incorporated the ω-alkynyl-palmitate analog in an alkali-sensitive manner as assessed by Western blot/ECL using NA-HRP. Of note, the detected signal was stronger in the dually palmitoylated EGFP-H-Ras than the singly palmitoylated EGFP-N-Ras, as could be predicted, suggesting that our method might not be only qualitative but could also be quantitative.

Upon optimization of the concentration of label to be added to COS-7 cells transiently transfected with EGFP-N-Ras (Fig. 4B), and the time of labeling at an optimal concentration of ω-alkynyl-palmitate analog (Fig. 4C), we found that incubation of transfected COS-7 cells with 100 μM ω-alkynyl-palmitate-BSA conjugate for 3 h was optimal. As shown in Fig. 4B, the acylation of EGFP-N-Ras with ω-alkynyl-palmitate can be readily detected after Cu^+-catalyzed azide-alkyne cycloaddition/biotinylation, electrophoretic separation, and Western blot/ECL/NA-HRP (5 s exposure) in lanes containing 25 μg of protein from whole cell lysate. In this case, incorporation of the analog was so significant that no immunoprecipitation of Ras was required to enrich the sample. Interestingly, incorporation of ω-alkynyl-palmitate into EGFP-N-Ras was alkali sensitive as expected, but that of 25, 37, 39, 41, 60, and 76 kDa proteins (highlighted by asterisks in the figure) was resistant to alkali treatment. The latter 76 kDa protein migrated just under an endogenously biotinylated protein. Also noteworthy, a faint band incorporating the ω-alkynyl fatty acid in an alkali-resistant manner comigrating with the EGFP-Ras protein can be seen in the untransfected control. Whether these proteins incorporated the

ω-alkynyl-palmitate via an amide bond at an N-terminal glycine residue or on a lysine residue, or whether the ω-alkynyl-palmitate was converted into ω-alkynyl-myristate prior to incorporation is under investigation. To further highlight the detection enhancement achieved by our protocol, in Fig. 4C, we show that the detection of acylated transiently expressed EGFP-N-Ras can be achieved with a labeling period of only 1 min using 100 μM ω-alkynyl-palmitate-BSA conjugate (shown as time = 0, Fig. 4C). These surprising results promise to greatly facilitate the study of acylation/deacylation kinetics using pulse-chase type experiments.

To further assess the labeling specificity using fatty acid analogs of various chain lengths, we compared the incorporation of various ω-alkynyl-fatty acids (C14, C16, and C18) into palmitoylatable EGFP-H-Ras or EGFP-N-Ras to that of non-palmitoylatable EGFP-K-Ras, in appropriately transfected COS-7 cells. In **Fig. 5A**, we show that the 16C ω-alkynyl-palmitate is preferentially and readily (2 s exposure to film) incorporated into EGFP-H-Ras, but not EGFP-K-Ras, in

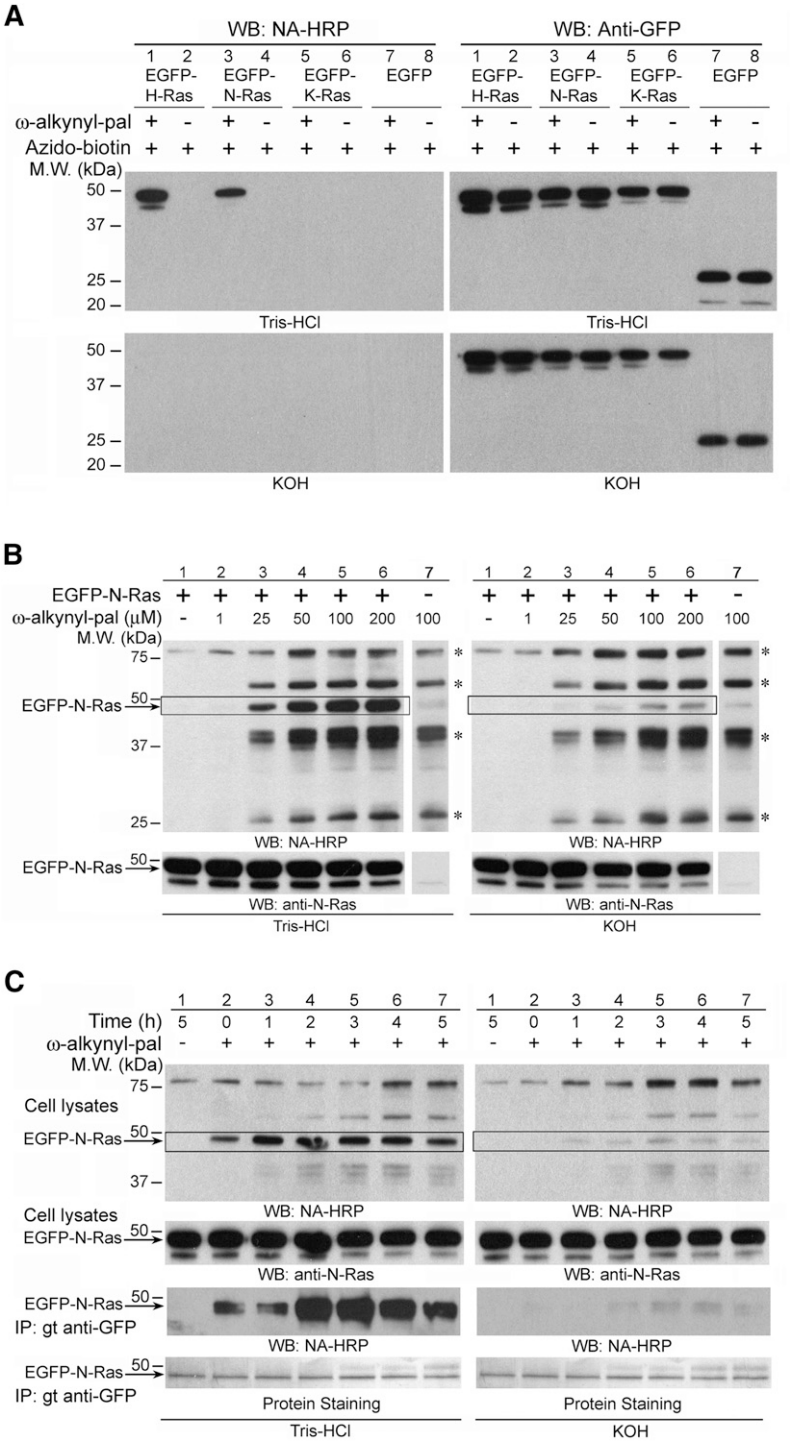

Fig. 4. Incorporation of ω-alkynyl-palmitate into proteins in COS-7 cells transiently expressing various EGFP-Ras chimeric proteins or EGFP at various label concentrations and time points. A: COS-7 cells transiently expressing EGFP-H-, N-, or K-Ras (lanes 1–6) or EGFP (lanes 7 and 8) were metabolically labeled with 100 μM ω-alkynyl-palmitate or DMSO vehicle as indicated for 3 h followed by solubilization, immunoprecipitation with anti-GFP, and reactions with azido-biotin using click chemistry. B: Lysates (25 μg per lane) of COS-7 cells transiently expressing EGFP-N-Ras (lanes 1–6) or without transfection (lane 7) metabolically labeled with increasing concentrations of ω-alkynyl-palmitate for 3 h. C: Lysates (15 μg per lane) of COS-7 cells transiently expressing EGFP-N-Ras metabolically labeled with 100 μM ω-alkynyl-palmitate for various periods of time as shown. Anti-GFP immunoprecipitations corresponding to lanes 1–7 are aligned below the main panels. Of note, for the zero time point (0), the BSA-conjugated ω-alkynyl-palmitate was added to the media and incubated for 1 min then removed by aspiration. Protein samples were reacted with azido-biotin using click chemistry, separated by electrophoresis, and processed for ECL. Prior to assessment of label incorporation by Western blotting/NA-HRP (1:50,000)/ECL, membranes were incubated in 0.1N Tris-HCl pH 7.0 (upper panels for A and left panels for B and C) or 0.1N KOH (lower panels for A and right panels for B and C). The right panels in A and insets in B and C show the similar expression/immunoprecipitation levels of the EGFP-Ras proteins as assessed by Western blotting using rabbit anti-GFP antibodies or rabbit anti-N-Ras antibodies.

comparison to the 14C ω-alkynyl-myristate or the 18C ω-alkynyl-stearate. The incorporation of ω-alkynyl-myristate (C14) or the ω-alkynyl-stearate (C18) into Ras protein did not come as a surprise, because protein fatty acyl transferases are known to have a broad substrate specificity. Indeed, in S-acylation, several LCFAs (e.g., C16:0, C16:1, C18:0, C18:1, and even C14:0) are often covalently attached to cysteine residues of proteins (9, 10). Labeling transfected COS-7 cells with the ω-azido-fatty acids (N3-C12, N3-C14, N3-C16) followed by copper(I)-catalyzed cycloaddition using an alkynyl-biotinylating compound analogous to our azido-biotin resulted in significantly higher background levels (data not shown), as described above and previously (41). In a similar fashion, the isosteric equivalent of myristate, the 14C ω-alkynyl-fatty acid, was efficiently incorporated into the myristoylatable ctPAK2-N_{15}-EGFP chimeric protein (Fig. 5B). However, the 16C ω-alkynyl-palmitate and 18C ω-alkynyl-stearate analogs were also incorporated into ctPAK2-N_{15}-EGFP, but to a much lesser extent. This can also be seen when using [^{3}H]palmitate after a 50 day exposure (Fig. 5C), but to a much lesser degree due to the significantly lower sensitivity of detection of the fluorographic process. Two possible explanations can account for this phenomenon; the longer chain fatty acids are undergoing β-oxidation to myristate and are subsequently incorporated into proteins by NMT or NMT can also accommodate these fatty acids with

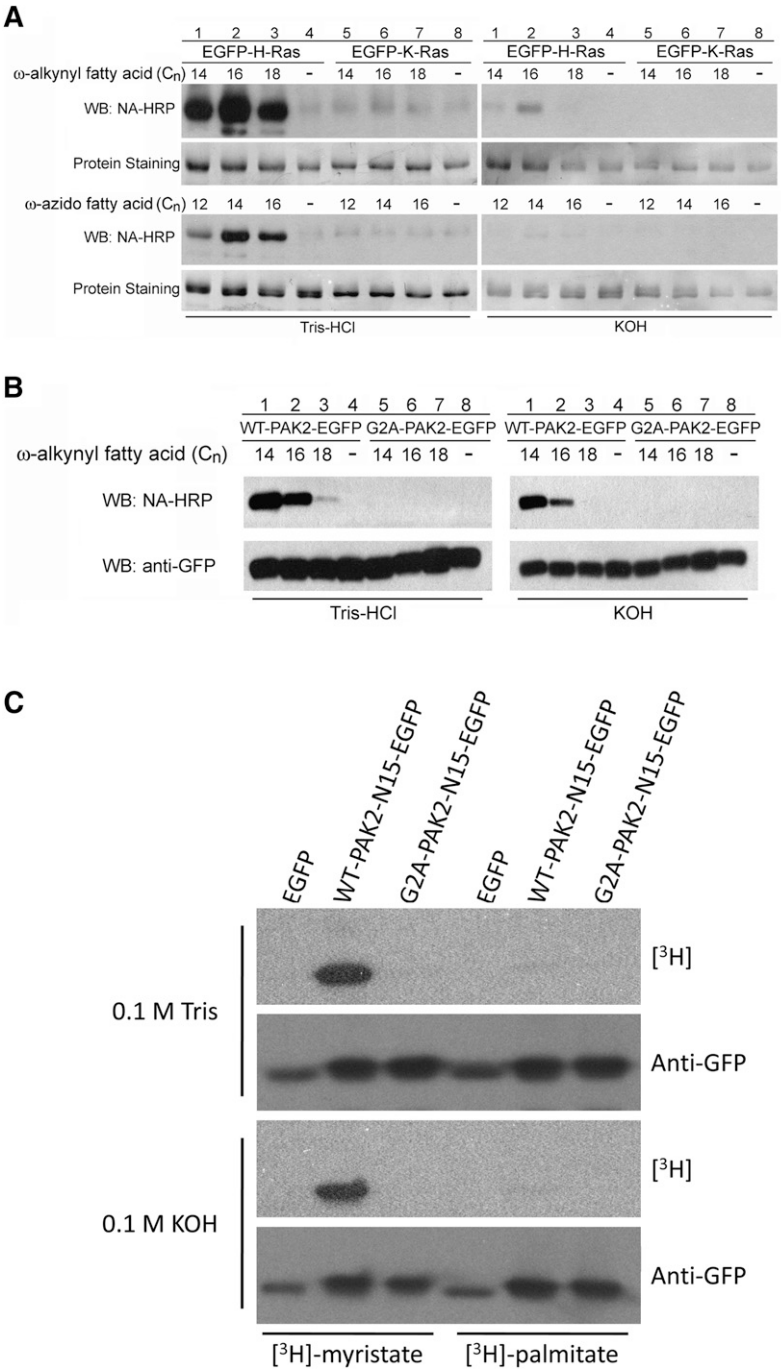

Fig. 5. Incorporation of ω-alkynyl-fatty acids analogs, ω-azido-fatty acid analogs, or tritiated fatty acids into proteins in COS-7 cells transiently expressing various EGFP tagged proteins. COS-7 cells transiently expressing palmitoylatable EGFP-H-Ras (A) or myristoylatable wild-type ctPAK2-N_{15}-EGFP (B) (lanes 1–4) and nonpalmitoylatable EGFP-K-Ras (A) or nonmyristoylatable mutant G2A-ctPAK2-N_{15}-EGFP (B) (lanes 5–8) chimeras were metabolically labeled with 100 μM of ω-alkynyl-myristate or ω-azido-myristate (lanes 1 and 5), ω-alkynyl-palmitate or ω-azido-palmitate (lanes 2 and 6), ω-alkynyl-stearate or ω-azido-stearate (lanes 3 and 7), or DMSO vehicle (lanes 4 and 8) for 3 h followed by solubilization, immunoprecipitation with anti-GFPs, and reacting with azido-biotin using click chemistry (top panels of A and B, exposure: 2 s) or phosphine-biotin using the Staudinger ligation (lower panels of A, exposure: 10 s). C: COS-7 cells transiently expressing EGFP, WT-ctPAK2-N_{15}-EGFP, or G2A-ctPAK2-N_{15}-EGFP were radiolabeled with 500 μCi of [^{3}H]myristate or [^{3}H]palmitate for 4 h. Immunocomplexes were separated by SDS-PAGE, blotted onto PVDF membranes, and detected by rabbit anti-GFP serum. PVDF membranes containing radiolabeled proteins were air-dried and exposed to Biomax MS film for 50 d at −80°C. Where indicated, membranes were incubated in 0.1 N Tris-HCl, pH 7.0, or 0.1 N KOH prior to processing and film exposure.

lower efficiency. Due to the lower incorporation efficiencies, these types of incorporation could not have been detected previously due to the lower detection sensitivity methods available (mainly incorporation of tritiated fatty acids). There was no incorporation of any fatty acids into the nonmyristoylatable form wherein the essential glycine was substituted to an alanine (G2A) (Fig. 5B, C).

To compare the labeling efficiency of ω-alkynyl fatty acids to that ω-azido-fatty acids, in a parallel experiment, we monitored the incorporation of the ω-N3-C12, ω-N3-C14, and ω-N3-C16 fatty acids (isosteric analogs of myristate, palmitate, and stearate, respectively) into EGFP-H-Ras and EGFP-K-Ras as described above but using the Staudinger ligation with a phosphine-biotin tag as a detection method as described in Martin et al. (16). As expected, we found that the ω-azido-tetradecanoate (ω-N3-C14), which is the most isosteric azido fatty acid analog of palmitate, was incorporated preferentially into EGFP-H-Ras, but not K-Ras (Fig. 5A). The signals obtained with the phosphine-biotin tag were less intense than those obtained using click chemistry and the exposure time was 5 times longer (10 s vs. 2 s). This suggests that using ω-alkynyl-fatty acids to label cells, in combination with the Cu^{+}-catalyzed azide-alkyne cycloaddition/biotinylation reaction, results in a 5- to 10-fold increase in sensitivity and is equally selective. Because of the background problems mentioned above using ω-azido-fatty acid followed by reaction with the alkynyl-biotin probe and the similar problems seen by others (32, 41), the click chemistry reaction in the reversed orientation was not investigated further.

When further addressing the selectivity of incorporation of various fatty acid analogs, results obtained in Fig. 5A were also corroborated when the competitive inhibitors of myristoylation and palmitoylation, HMA and 2-BP, were added to cells prior to labeling with ω-alkynyl-myristate or ω-alkynyl-palmitate, respectively (**Fig. 6**). When COS-7 cells, transiently expressing EGFP-N-Ras, were labeled in such a manner and solubilized with SDS followed by the Cu^{+}-catalyzed azide-alkyne cycloaddition/biotinylation reaction, we showed that the incorporation of ω-alkynyl-palmitate into EGFP-N-Ras was inhibited by 2-BP, but not HMA, in cell lysates and in immunoprecipitated EGFP-N-Ras (Fig. 6A, lanes 2–4, top and lower panels). Alkali treatment removed the ω-alkynyl-palmitate label attached to several proteins (e.g., EGFP-N-Ras, lanes 2 and 3, or that of a ~20 kDa protein, lanes 5 and 6) but not all. When COS-7 cells were transiently transfected with a reporter construct known to express the myristoylated protein ctPAK2-N_{15}-EGFP (15), incorporation of ω-alkynyl-myristate into ctPAK2-N_{15}-EGFP was blocked by HMA, but not 2-BP (Fig. 6B). Again, the signal was strong enough in the cell lysates that immunoprecipitation of the reporter protein was not required to visualize incorporation of the ω-alkynyl-fatty acid. In addition, we demonstrate in Jurkat-T cells that ω-alkynyl-myristate can be specifically incorporated into various endogenous proteins cotranslationally as well as endogenous caspase cleaved proteins posttranslationally in cells undergoing Fas-mediated apoptosis (Fig. 6C), and, more specifically, in the endogenous caspase-cleaved C-terminal PAK2 fragment (Fig. 6D). When Jurkat cells were labeled with ω-alkynyl-myristate in the presence of HMA or 2-BP or in their absence, the incorporation of ω-alkynyl-myristate into proteins was blocked by the presence of the NMT inhibitor HMA, but not the palmitoylation inhibitor 2-BP (data not shown). The incorporation of ω-alkynyl-myristate was resistant to alkali treatment, suggesting that ω-alkynyl-myristate is incorporated into proteins via an amide bond (data not shown). These data show that both ω-alkynyl-myristate and ω-alkynyl-palmitate analogs can be used to monitor myristoylation and palmitoylation of exogenously and endogenously expressed proteins in cultured cells.

As a complement to our labeling specificity study, we then sought to investigate whether incorporation of ω-alkynyl-palmitate into a known palmitoylated protein would interfere with its subcellular localization. Using differential centrifugation as a means to obtain nuclear (P1), mitochondrial/microsomal "heavy" membrane (P10), microsomal "light" membrane (P100), and cytosolic (S100) fraction as we described in Vilas et al. (40), we showed that labeling of COS-7 cells transiently expressing EGFP-N-Ras with ω-alkynyl-palmitate does not affect the subcellular fractionation pattern of palmitoylated EGFP-N-Ras. A weak signal found in the S100 fraction likely corresponds to the nonspecific band shown in the control experiment containing unlabeled palmitate or an endogenous acylated protein or, alternatively, the residual fatty acylated EGFP-N-Ras found in the S100 fraction could be the result of some nonspecific transfer of loosely packed membranes during our differential centrifugation fraction protocol. Whether cells were labeled with palmitate or ω-alkynyl-palmitate, EGFP-N-Ras labeled with ω-alkynyl-palmitate was predominantly found in the membrane fractions (P1, P10, P100) (**Fig. 7**). Furthermore, the overall distribution of EGFP-N-Ras among fractions was not altered by the addition of ω-alkynyl-palmitate to cells prior to fractionation. This suggests that the membrane binding function of EGFP-N-Ras is not affected when it is acylated with ω-alkynyl-palmitate analog.

Palmitoylation of endogenous H- and N-Ras in cultured cell lines and in vivo (in mice) is readily detected using ω-alkynyl-palmitate and click chemistry

To fully assess the detection sensitivity of our labeling protocol using ω-alkynyl-palmitate, we then proceeded to measure acylation of endogenous Ras proteins in cultured cell lines and in vivo in mice (**Fig. 8**). In Fig. 8A, we show that ω-alkynyl-palmitate is readily incorporated into endogenous H-/N-Ras proteins via an alkali labile bond from HeLa and Jurkat T cells as detected after immunoprecipitation using anti-Ras antibodies, Cu^{+}-catalyzed cycloaddition using azido-biotin, and Western blotting/ECL with NA-HRP. To assess protein palmitoylation in vivo for the very first time, we injected 200 μl of a BSA conjugated 1 mM ω-alkynyl-palmitate or palmitate (10× stock solution) into the tail vein of fasted BALB/C mice. One or four hours postinjection, animals were euthanized and their livers were excised. Following im-

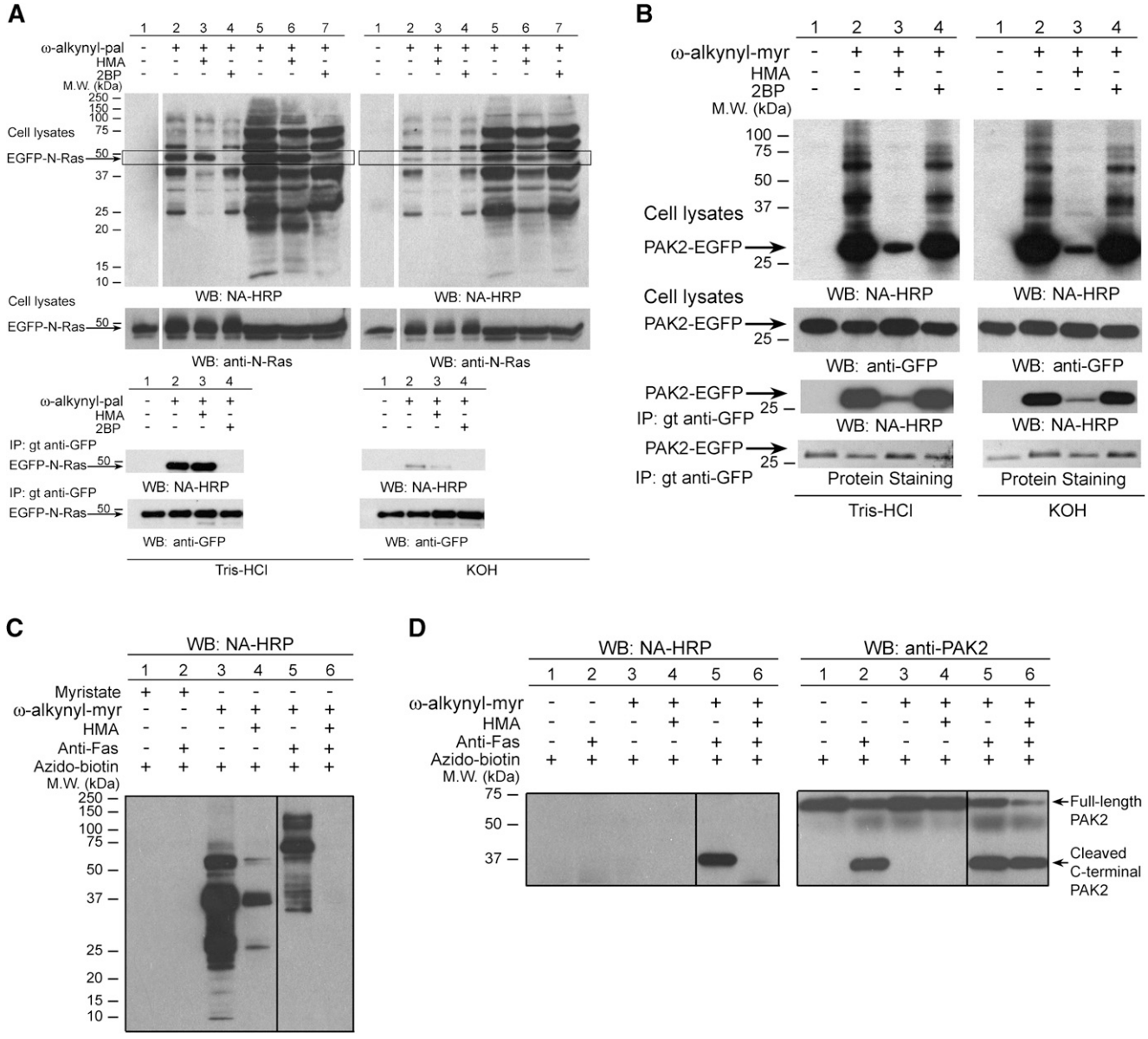

Fig. 6. Incorporation of ω-alkynyl-fatty acid analogs into palmitoylated proteins and co- or posttranslationally myristoylated proteins is sensitive to selective inhibition by competitive inhibitors 2-BP and HMA, respectively. COS-7 cells transiently expressing EGFP-N-Ras (A) or WT-ctPAK2-N_{15}-EGFP (B) were incubated with the N-myristoylation inhibitor, HMA (1 mM), the palmitoylation inhibitor 2-BP (100 μM), or DMSO vehicle as indicated for 1 h prior to metabolic labeling with DMSO vehicle (lane 1) or 100 μM ω-alkynyl-palmitate (lanes 2–7) (A) or 25 μM ω-alkynyl-myristate (lanes 2–4) (B) for 3 h. Lanes 1–4 of main panels of A and B: 10 μg cell lysate/lane; lanes 5–7 of main panels of A: 50 μg cell lysate/lane. Anti-GFP immuno-precipitations corresponding to lanes 1-4 are aligned below the main panels of A and B. In panels C and D, untransfected Jurkat T cells were incubated with 1 mM HMA or sodium myristate for 1 h prior to metabolic labeling with 25 μM of myristate (lanes 1 and 2) or ω-alkynyl-myristate (lanes 3–6) for 1 h. For induction of apoptosis (lanes 2, 5, and 6), 150 ng/ml of anti-Fas antibody and 5 μg/ml of cycloheximide were added to the cells for 6 h prior to cell harvesting. Endogenous PAK2 was immunoprecipitated by rabbit anti-ctPAK2 antibody and detected by goat anti-ctPAK2 (C19) antibody (D). Where indicated, membranes were incubated in 0.1 N Tris-HCl, pH 7.0, or 0.1 N KOH prior to processing and film exposure.

munoprecipitation of Ras proteins from a solubilized liver homogenate, we demonstrated the incorporation of ω-alkynyl-palmitate via an alkali labile thioester bond after click chemistry using azido-biotin and detection by Western blotting/ECL at both time points (Fig. 8B). Although there is some background in the control lanes, the signal corresponding to the samples originating from animals injected with the ω-alkynyl-fatty acid were clearly stronger. Assuming a 2 ml blood volume for BALB/C mice when using an approximate 100 μM circulating concentration of ω-alkynyl-palmitate, we could only detect palmitoylation after immunoprecipitation and concentration of the Ras proteins. Some of the background might originate from the nonspecific binding of NA-HRP to the immunoprecipitated Ras protein (>1 μg/lane). We are currently optimizing delivery and uptake kinetics of the ω-alkynyl-palmitate analog into mice. To our knowledge, this represents the first direct demonstration of protein palmitoylation in vivo.

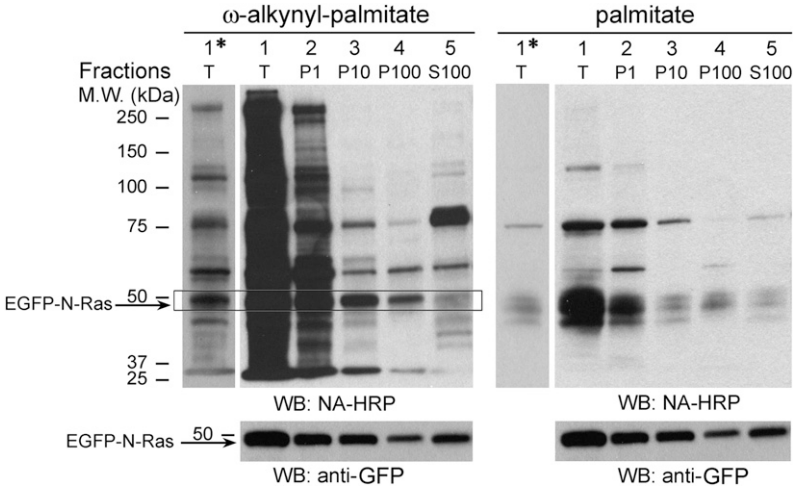

Fig. 7. Labeling COS-7 cells transiently expressing EGFP-N-Ras with ω-alkynyl-palmitate does not interfere with the subcellular localization of EGFP-N-Ras. COS-7 cells transiently expressing EGFP-N-Ras metabolically labeled with 100 μM ω-alkynyl-palmitate (left) or palmitate (right) for 3 h were subjected to subcellular fractionation by differential centrifugation to yield P1, P10, P100, and S100 fractions as described in Materials and Methods (20% of each fraction was loaded in each lane). Protein fractions were reacted with azido-biotin using click chemistry, separated by electrophoresis, and processed for ECL. Lane 1: 5 s film exposure; lanes 1–5: 1 min film exposure. Lower panels show the distribution of EGFP-N-Ras in different subcellular fractions.

DISCUSSION

Progress in understanding the biology of protein fatty acylation has been impeded by the lack of rapid direct detection and identification methods. The recent emergence of bio-orthogonal molecules that mimic natural substrates has advanced our repertoire of tools available for the study of posttranslational modifications (42). Recently, we and others reported the use of the isosteric myristate and palmitate analogs, ω-azido-tetradecanoic acid and ω-azido-dodecanoic acid, respectively, to label fatty acylated proteins in vitro using the Staudinger ligation for identification purposes (16, 20, 21). In an effort to improve on the published methodology (16, 20, 21), to minimize the potential dipole effects of the azide moiety linked to the fatty acid, and to utilize a fatty acid analog that would better maintain the physiological characteristics of palmitate and myristate, we and others (26, 34, 41) successfully investigated the potential of ω-alkynyl-fatty acids with click chemistry using azido-probes as a means to detect fatty acylation of proteins.

Using a competition assay and MS, we demonstrated that the incorporation of the ω-alkynyl-palmitoyl moiety occurs at the same site as palmitate in vitro on two metabolic enzymes, HMGCS and GAPDH. Using ω-alkynyl-palmitoyl-CoA to acylate HMGCS followed by MS analysis, we showed that ω-alkynyl-palmitate is incorporated on Cys305 as we have shown for palmitate and ω-azido-palmitate (unpublished observations). The role of palmitoylation of HMGCS is under active investigation. In addition, ω-alkynyl-palmitate was also readily imported into rat liver mitochondria in vitro, resulting in the palmitoylation of numerous mitochondrial proteins.

The surprising abundance of acylated proteins found in the mitochondria (23, 24), and the fact that the mitochondrial enzymes methylmalonyl semialdehyde dehydrogenase and carbamoyl phosphate synthetase 1 are inhibited by active site fatty acylation, opens up the exciting possibility that the dynamic fatty acylation of mitochondrial proteins might represent a novel and meaningful addition to the repertoire of cellular control mechanisms. The large number of these palmitoylated metabolic enzymes and the acylation-dependent inhibition of some of these catabolic enzymes suggest a key and underappreciated role for LCFA-CoAs in the regulation of intermediary metabolism. Even with the recent identification of 21 palmitoylated mitochondrial proteins by our group (21), a large majority of the palmitoylated mitochondrial proteins remains to be identified and characterized. Using the ω-alkynyl-palmitate analog and click chemistry will facilitate this task.

To evaluate the use of ω-alkynyl-palmitate in living systems, we took advantage of the fact that in addition to being isoprenylated and carboxymethylated, the H- and N-Ras (but not K-Ras) human proto-oncogenes are also palmitoylated. We demonstrated that ω-alkynyl-palmitate is also selectively incorporated into H- and N-Ras isoforms and that ω-alkynyl-palmitate is specifically and preferentially incorporated into exogenously and endogenously expressed H- and N-Ras proteins via an alkali labile thioester bond in cultured cells and for the first time in vivo (in mice). Furthermore, labeling cells with ω-alkynyl-palmitate does not affect the subcellular fractionation pattern of N-Ras. By showing it does not interfere with membrane insertion of N-Ras, some potential concerns of using an ω-alkynyl-palmitate as a chemical reporter for the study of protein palmitoylation are alleviated. In addition, we demonstrated that ω-alkynyl-myristate is also specifically incorporated co- and posttranslationally into several endogenous proteins, including caspase-cleaved ctPAK2. Incorporation of the ω-alkynyl-myristate analog was inhibited by the NMT inhibitor HMA or blocked by mutating the essential N-terminal Gly to Ala into exogenously expressed ctPAK2-N_{15}-EGFP, thereby demonstrating that like myristate, it can be selectively incorporated into myristoylated proteins by NMTs.

Whether used to monitor the labeling of acylated protein in vitro, in cultured cell lysates, or in immunoprecipitates, the click chemistry reactions between ω-alkynyl-fatty acylated proteins and azido-biotin readily occurred in vitro within 30 min at 37°C and allowed the detection of biotinylated-ω-alkynyl-fatty acylated proteins typically within seconds of film exposure when Western blotting with NA-HRP (1:50,000) and ECL. The use of an ω-azido-myristate analog and phosphine-biotin

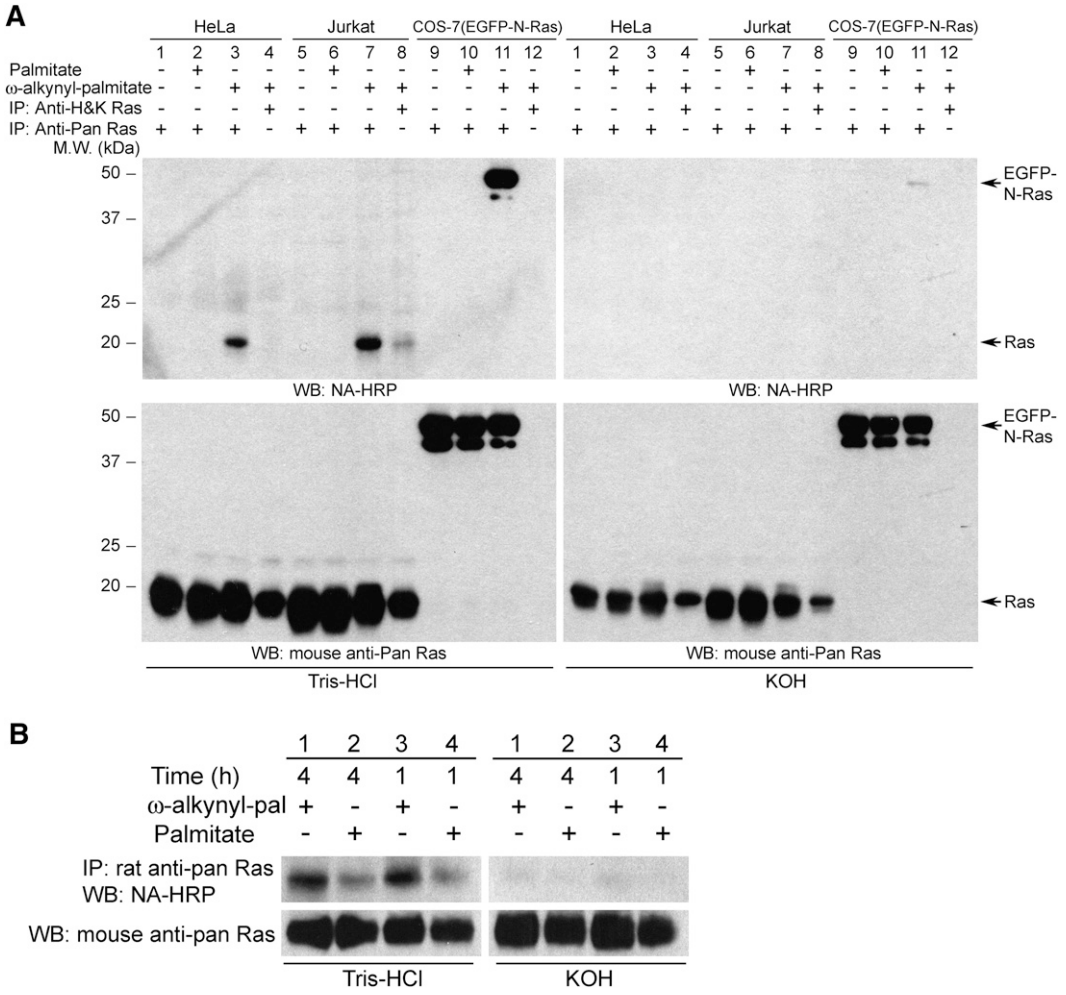

Fig. 8. Detection of endogenous Ras palmitoylation using ω-alkynyl-palmitate labeling in cultured Hela and Jurkat T cells and in vivo from mouse liver. A: Cultured Hela cells (lanes 1–4), Jurkat cells (lanes 5–8), or COS-7 cells transiently expressing EGFP-N-Ras as a positive control (lanes 9–12) were labeled with DMSO vehicle (lanes 1, 5, 9), palmitate (100 μM) (lanes 2, 6, 10), or ω-alkynyl-palmitate (100 μM) (lanes 3, 4, 7, 8, 11, 12) for 3 h. H- and K-Ras proteins were immunoprecipitated from solubilized cell lysates with rat anti-H- and K-Ras 238 antibody (lanes 4, 8, 12). Alternatively, immunoprecipitations were carried out with rat anti-pan-Ras 259 antibody. Immunoprecipitates were reacted with azido-biotin using click chemistry, separated by electrophoresis, and processed for ECL. B: Balb/c mice were injected in the tail vein with ω-alkynyl-palmitate or palmitate conjugated to BSA to yield an approximate 100 μM circulating concentration of the fatty acids. At 1 and 4 h postinjection, Ras proteins were immunoprecipitated from solubilized liver homogenates with rat anti-pan-Ras 259 antibody. Immunoprecipitates were reacted with azido-biotin using click chemistry, separated by electrophoresis, and processed for ECL. Prior to assessment of label incorporation by Western blotting/NA-HRP (1:50,000)/ECL, membranes were incubated in 0.1 N Tris-HCl, pH 7.0 (left panels), or 0.1 N KOH (right panels). The presence of the Ras proteins on membranes was assessed by Western blotting using mouse anti-pan-Ras antibody.

to detect posttranslational myristoylation of caspase-cleaved proteins led to a million-fold increase in detection sensitivity when compared with using tritiated myristate as label followed by fluorography (16, 20). When we compared the efficiency of click chemistry with ω-alkynyl-palmitate to the Staudinger ligation with ω-azido-palmitate (Fig. 5A), the detection of ω-alkynyl-palmitoylated Ras using click chemistry gave at least twice the signal in a 5-time shorter exposure, thereby adding another order of magnitude in detection sensitivity. Thus, by comparison, the use of click chemistry with ω-alkynyl-palmitate as label and azido-biotin as probe offers an approximate 7 order of magnitude improvement on the use of tritiated fatty acid as labels.

In our in vitro acylation reactions, followed by click chemistry using the fluorogenic probe 3-azido-7-hydroxycoumarin and separation of the products by SDS-PAGE, detection of the labeled proteins could be achieved immediately following SDS-PAGE with a flat bed fluorescence scanner. The reaction of 3-azido-7-hydroxycoumarin with alkynes forms a fluorescent 1,2,3-triazole ligation product, whereas the nonreacted 3-azido-7-hydroxycoumarin remains nonfluorescent (43). This characteristic combined with the ability to scan the gels for fluorescence immediately

following SDS-PAGE makes this method of labeling a highly valuable tool and would also allow us to readily obtain acylation protein profiles (32, 44) for fatty acylated proteins in metabolically compromised and normal cells. This is the first example of the successful use of the ω-alkynyl fatty acids with the activatable fluorophore 3-azido-7-hydroxycoumarin. The fact that this compound has the added benefit of becoming fluorescent only after the specific reaction with alkyne analogs promises to be a useful tool and alternative to follow fatty acylation in cells by microscopy (26, 34, 41).

Because the expression levels of the two human NMTs (45) and several zinc finger, Asp-His-His-Cys-PATs (e.g., zDHHC-2, -11, and -14) are altered in several types of tumors (46–48), ω-alkynyl-myristate, and ω-alkynyl-palmitate analogs could be both utilized to assess myristoylation and palmitoylation patterns in normal and cancer cells. This should therefore facilitate the generation of "acylation"-based protein profiles, the identification of fatty acylated proteins specific to cancer cells, and the characterization of the contributions of the various acylated proteins and acyl transferases to the metastatic process. The use of ω-alkynyl-fatty acids in animals could also eventually facilitate the identification of abnormally fatty acylated proteins in tissues of metabolically compromised model animals. Facilitating such studies is the fact that ω-azido-fatty acid analogs were shown to be practically inert and harmless to cells or animals (49). Due to the stability of the triple bond in biological systems, we believe ω-alkynyl-fatty acids are equally harmless in animals, as supported by our very preliminary studies and as is being further addressed experimentally in mice in our laboratory.

The use of ω-alkynyl-palmitate will provide an invaluable tool for proteomic studies of protein palmitoylation in cultured cells and in vivo. Like the acyl-biotin exchange reaction (50), our labeling protocol using click chemistry results in biotin-tagged proteins, which can be affinity-purified using avidin-agarose technologies and protein identifications made by proteomic MS, as recently described (26). In addition to a large variety of commercially available avidin-agarose beads, the recent development of agarose-immobilized azide groups (51) could facilitate the isolation and identification of ω-alkynyl-acylated proteins from cells or animal tissues.

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