Heterogeneous Fatty Acylation of Src Family Kinases with Polyunsaturated Fatty Acids Regulates Raft Localization and Signal Transduction*

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Fatty acylation of Src family kinases is essential for localization of the modified proteins to the plasma membrane and to plasma membrane rafts. It has been suggested that the presence of saturated fatty acyl chains on proteins is conducive for their insertion into liquid ordered lipid domains present in rafts. The ability of unsaturated dietary fatty acids to be attached to Src family kinases has not been investigated. Here we demonstrate that heterogeneous fatty acylation of Src family kinases occurs and that the nature of the attached fatty acid influences raft-mediated signal transduction. By using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, we show that in addition to 14:0 (myristate), 14:1 and 14:2 fatty acids can be attached to the N-terminal glycine of the Src family kinase Fyn when the growth media are supplemented with these dietary fatty acids. Moreover, we synthesized novel iodinated analogs of oleate and stearate, and we showed that heterogeneous S-acylation can occur on cysteine residues within Fyn as well as Go, GAP43, and Ras. Modification of Fyn with unsaturated or polyunsaturated fatty acids reduced its raft localization and resulted in decreased T cell signal transduction. These studies establish that heterogeneous fatty acylation is a widespread occurrence that serves to regulate signal transduction by membrane-bound proteins.

A growing number of viral and cellular proteins have been shown to be modified by covalent attachment of the fatty acids myristate and/or palmitate. Myristate is co-translationally attached to the N-terminal glycine through an amide linkage, whereas palmitate is attached post-translationally to proteins via a thioester linkage (S-acylation). There is increasing evidence in the literature suggesting that protein fatty acylation is not restricted to myristate and palmitate. For example, the retinal proteins recoverin and transducin have been shown to be heterogeneous fatty acylated via amide linkage with 12:0, 14:1, and 14:2 fatty acids in addition to 14:0 myristate (1–3). However, analysis of the amide-linked fatty acids from total proteins in heart, liver, brain, and retina indicated that heterogeneous myristoylation is restricted to the retina (2, 4).

S-Acylation of proteins is more diverse, and numerous reports have shown that some proteins are S-acylated with fatty acids longer and shorter than palmitate. For example, stearate, oleate, arachidonate, and eicosapentaenoate have been shown to be acylated to “palmitoylated” proteins in platelets as well as other cell types (5). Analysis of S-linked fatty acids released from total heart and liver proteins reveals the presence of detectable amounts of 14:0, 18:0, 18:1, and 18:2 fatty acids in addition to 16:0 (4). Moreover, the pool of fatty acids covalently bound to platelet proteins via thioester linkages can be altered by exogenously supplied fatty acids (6). Whether S-acylation with different fatty acids affects protein localization and function has not yet been elucidated.

A number of palmitoylated signaling molecules, such as Src family kinases, G proteins, and endothelial nitric-oxide synthase are enriched in membrane microdomains known as rafts that are resistant to low temperature extraction by non-ionic detergent. Palmitoylation has been shown to be required for localization of these signaling molecules to rafts (7–9). Many of the critical signaling components involved in T cell receptor-mediated signaling are localized to rafts, including Fyn, Lck, and LAT (10). Activation of the T cell receptor results in recruitment of other signaling molecules to rafts, including the ζ chain of the T cell receptor, ZAP-70, Vav, and phospholipase CyI (11, 12). The importance of rafts for T cell receptor signaling is illustrated by the finding that disruption of raft structure by agents such as filipin or nystatin inhibits early steps of T cell receptor activation. Moreover, mutation of the palmitoylation sites within LAT prevents LAT partitioning to rafts and consequently inhibits the recruitment of LAT-binding proteins such as Vav and phospholipase CyI to rafts (13). These data imply that partitioning of proteins into rafts enhances the protein/protein and protein/lipid interactions that are critical for efficient signal transduction in T cells.

A recent study reported that incubation of T cells with polyunsaturated fatty acids (PUFAs) inhibits T cell receptor-mediated signal transduction by displacing Lck and Fyn from rafts (14). Our laboratory then showed that PUFAs inhibit Fyn

1 The abbreviations used are: 18:0, stearic acid; 18:1, oleic acid; 16:0, palmitic acid; 20:4, arachidonic acid; 20:5, cis-5,7,11,14,17-eicosapentaenoic acid; IC13, 13-isododecanedioic acid; IC16/0, 16-isohexadecanedioic acid; IC18:1, 18-isooctadecanedioic acid; IC18:0, 18-iodo-octadecanedioic acid; PAGE, polyacrylamide gel electrophoresis; MAP, mitogen-activated protein; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; NMT, N-myristoyltransferase; MALDI-TOF MS, matrix-assisted laser desorption/ionization reflectron time-of-flight mass spectrometry; PUFAs, polyunsaturated fatty acids; DMEM, Dulbecco’s modified Eagle’s medium; eGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; BSA, bovine serum albumin; TEV, Tobacco Etch Virus; TCR, T cell receptor.
palmityloylation and localization to rafts in COS-1 cells (15). We postulated that S-acylation of Fyn with PUFA s was occurring and thereby altering Fyn localization within the plasma membrane. Lipids with unsaturated fatty acid chains are excluded from rafts, and it is likely that proteins acylated with unsaturated fatty acids may also exhibit altered association with these specialized membrane microdomains. Here, we investigated how dietary fatty acids affect fatty acylation, membrane localization, and signal transduction capacity of Src family kinases. The aims of the present study were as follows: 1) to determine whether Src family kinases can be acylated with fatty acids other than myristate and palmate; and 2) to determine how differential fatty acylation influences the ability of Src family kinases to interact with the membrane and other signal transducing molecules. Here we show that the Src family member Fyn can be heterogeneously N-myristoylated with 14:1 and 14:2 fatty acids in addition to 14:0 myristate. In addition, we show that Fyn can also be heterogeneously S-acylated with dietary fatty acids other than palmitate, including oleic acid and arachidonic acid. Moreover, incorporation of unsaturated fatty acids displaces Fyn from membrane rafts, which prevents it from interacting with other signaling molecules and results in the inhibition of signal transduction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Palmitic acid, oleic acid, arachidonic acid, and cis-5,8,11,14,17-eicosapentaenoic acid were purchased from Sigma. 5-cis-Tetradecenoic acid and 5-cis,8-cis-tetradecadienoic acid were kindly provided by Dr. Horst Schulz. [1H]Palmitic acid, [1H]oleic acid, and [1H]arachidonic acid were purchased from PerkinElmer Life Sciences. Recombinant Tev protease and Optiprep gradient medium were purchased from Life Technologies, Inc. RPS-F TLC plates used for fatty acid analysis were purchased from Analtech (Newark, DE). Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen (Valencia, CA). The synthesis of α-iodoteicadecanoic acid and α-iodoteicadecanoic acid was carried out by Dr. William Bornmann at the Core Chemistry Facility of Memorial Sloan-Kettering Cancer Center. Synthesis methods are available upon request.

**Antibodies**—Monoclonal anti-pErk, rabbit polyclonal anti-Ha-Ras, and polyclonal anti-caveolin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Fyn monoclonal antibody was from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-LAT antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-Fyn was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antisera to Fyn used for immunoprecipitation was described previously (16). OKT3 monoclonal antibody was obtained from the Monoclonal Antibody Core Facility at Massachusetts General Hospital (Boston, MA). Anti-8-cis-14:0 Fyn antibody was from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-Ha-Ras, rabbit polyclonal anti-LAT, rabbit polyclonal anti-Fyn antibody was from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-LAT antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-Fyn was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antisera to Fyn used for immunoprecipitation was described previously (16). OKT3 monoclonal antibody was obtained from the Monoclonal Antibody Core Facility at Massachusetts General Hospital (Boston, MA). Anti-8-cis-14:0 Fyn antibody was from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-LAT antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-Fyn was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antisera to Fyn used for immunoprecipitation was described previously (16). OKT3 monoclonal antibody was obtained from the Monoclonal Antibody Core Facility at Massachusetts General Hospital (Boston, MA). Anti-8-cis-14:0 Fyn antibody was from New England Biolabs (Beverly, MA).

**Cell Culture, Plasmids, and Transfections**—COS-1 cells and Jurkat T cells were maintained as described previously (16). COS-1 cells were transfected with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. The Fyn chimeras Go(10-F), Gap43(10-F), Fyn, G2A, C3SFy-n-Ha-Ras, and G12V-Ha-Ras have been described previously (16). C3SFy-n-Ha-Ras and C3SFy-n-Ha-Ras have been described previously (16). Cells were homogenized in hypotonic buffer and centrifuged at 100,000 × g for 45 min to obtain supernatants (S100) and pellets (P100) as described previously (14). Immunoprecipitation was performed in RIPA buffer with anti-Fyn antibody as described previously (16).

**Isolation of Rafts from Optiprep Density Gradients**—Isolation of Triton X-100-insoluble membrane fractions was carried out essentially as described (20). Fatty acids were first dissolved in ethanol, and ethanol was then removed under N2. A 2.5% BSA solution containing growth medium and 2% dialyzed FBS was added to the fatty acids, and the suspension was sonicated for 2 min to generate a BSA-fatty acid complex. COS-1 cells expressing wild type Fyn were incubated for 12 h in DMEM containing 2% dialyzed FBS and 0.25% defatted BSA supplemented with either 100 μM myristic acid or 5-cis,8-cis-tetradecadienoic acid. Cells were homogenized in hypotonic buffer and centrifuged at 100,000 × g for 45 min. The supernatant (S100) and pellets (P100) were collected from the top to the bottom. RIPA buffer (5×) was added to solubilize the pellets. After centrifugation, proteins were concentrated by precipitation with 20% trichloroacetic acid and analyzed by SDS-PAGE and Western blotting.

To study raft localization of fatty acid radiolabeled Fyn, COS-1 cells expressing Fyn were incubated with 25–50 μCi/ml of either 16:1-docosahexaenoic acid, 18:4-ω3-docosahexaenoic acid, 18:3-ω3-docosahexaenoic acid, or 18:2-ω6-docosahexaenoic acid in DMEM containing 2% dialyzed FBS and 0.25% defatted BSA as described above. The cells were washed and scraped off the plate with STE buffer and subjected to the raft isolation procedure as described above. Fractions were collected and subjected to immunoprecipitation with anti-Fyn antibody, followed by SDS-PAGE and Western blotting or phosphorimaging.

**Activation of MAP Kinases**—Jurkat T cells were incubated for 50 μM of either stearic acid, arachidonic acid, cis-5,7,11,14,17-eicosapentaenoic acid in RPMI medium containing 2% dialyzed FBS and 0.25% defatted BSA. Cells were washed with STE (150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA) and lysed in 300 μl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM Na3VO4) supplemented with protease inhibitors for 15 min in ice. After centrifugation, 200 μl of the supernatant was overlaid with 3.5 ml of 30% Optiprep to make a final density of 1.100 g/ml. After centrifugation at 170,000 × g for 3 h in an SW55 rotor, five fractions were collected from the top to the bottom. RIPA buffer (5×) was added to solubilize the pellets. After clarification by centrifugation at 100,000 × g for 15 min, proteins were concentrated by precipitation with 20% trichloroacetic acid and analyzed by SDS-PAGE and Western blotting.
kinase by immunoblotting with a polyclonal anti-MAP kinase antibody. **Fatty Acid Analysis by TLC**—Analysis of covalently bound fatty acids was performed as described (19). Briefly, COS-1 cells expressing Fyn were labeled with either 16-[^125]Iiodohexadecanoic acid, 18-[^125]Iiodo-20:0, or 18-[^125]Iiodo-22:2, as described above. Following immunoprecipitation of cell lysates with anti-Fyn antibody and electrophoresis, the wet gel slice containing the Fyn band was excised and treated with 1.5 M sodium hydroxide overnight at room temperature. After acidification, the mixture was extracted with chloroform twice. The combined chloroform extracts were concentrated under nitrogen and analyzed by reversed phase thin layer chromatography. The TLC plate was developed with 1:1.75:1.75 water-glacial acetic acid/acetonitrile. Following air drying, the plate was analyzed by phosphorimaging.

**Release of Protein-bound Fatty Acids by Hydroxylamine Treatment**—COS-1 cells expressing GAP43(10):Fyn chimeras were labeled with either 16[^125]Iiodohexadecanoic acid, 18[^125]Iiodo-20:0, or 18[^125]Iiodo-22:2 as described above. Following immunoprecipitation of cell lysates with anti-Fyn antibody, the samples were electrophoresed on two separate gels. After electrophoresis, the gels were first fixed in a 50% ethanol, 5% acetic acid for 45 min and then washed with water. One gel was treated overnight with 2 M hydroxylamine (pH 7.0) and the other was treated with 2 M Tris-HCl (pH 7.0). The gels were rinsed with water, dried, and analyzed by phosphorimaging.

**RESULTS**

**Src Family Kinases Are Heterogeneously N-Myristoylated**—Studies of protein N-myristoylation have shown that in addition to 14:0 myristate, heterogeneous fatty acylation with 12:0, 14:1, and 14:2 fatty acids can occur on retinal proteins but not on proteins isolated from heart and liver. However, no study to date has directly examined whether Src family kinases can be N-myristoylated with fatty acids other than myristate. This question is important to address because acylation with unsaturated fatty acids could potentially alter the membrane localization and signaling properties of Src family kinases. We therefore designed a construct in which N-terminal acylation of the Src family kinase Fyn could be directly monitored using mass spectrometric techniques. First, the N-terminal 16 amino acids of Fyn were fused to eGFP. Cysteine residues at positions 3 and 6 of Fyn were mutated to serine in order to limit fatty acylation to the N-terminal glycine residue. We have previously shown that (C3,6S)Fyn incorporates myristate as efficiently as wild type Fyn. Next, the 7-amino acid recognition sequence of Tobacco Etch Virus (TEV) protease was inserted between residue 16 of Fyn and the eGFP sequence, in order to generate a unique cleavage sequence near the Fyn N terminus. Finally, the (C3,6S)Fyn16TevEGFPsi6 construct was tagged at the C terminus with 6 histidines in order to facilitate purification. N-terminal acylation of the Src family kinase Fyn could be directly monitored using mass spectrometric techniques. First, the N-terminal 16 amino acids of Fyn were fused to eGFP. Cysteine residues at positions 3 and 6 of Fyn were mutated to serine in order to limit fatty acylation to the N-terminal glycine residue. We have previously shown that (C3,6S)Fyn incorporates myristate as efficiently as wild type Fyn. Next, the 7-amino acid recognition sequence of Tobacco Etch Virus (TEV) protease was inserted between residue 16 of Fyn and the eGFP sequence, in order to generate a unique cleavage sequence near the Fyn N terminus. Finally, the (C3,6S)Fyn16TevEGFPsi6 construct was tagged at the C terminus with 6 histidines in order to facilitate purification.

**FIG. 1. Mass spectra of N-terminal acylated peptides of Fyn chimera.** A, design of the Fyn chimera used for mass spectrometric analysis. B–E, COS-1 cells expressing Fyn chimera were incubated with DMEM supplemented with various fatty acids. The protein was purified and digested with TEV protease. The cleaved peptides were analyzed by mass spectrometry. B, cells were incubated with or without tetradecanoic acid (C14:0). C, cells expressing G2A mutant of Fyn chimera were incubated with C14:0. D and E, cells were incubated with 5cis-tetradecenoic acid (C14:1) (D) or 5 cis,8-cis-tetradecadienoic acid (14:2) (E), respectively.

**A Construct**

\[(C3,6S)Fyn16\]Tev eGFP

\[\text{his6}\]

**B) C14 acid**

2625.44

**C) G2A mutant**

2471.19

**D) 5-cis-C14 acid**

2625.27

**E) 5-cis,8-cis-C14 acid**

2621.17

**m/z**

2623.28

2625.15

this mutant was analyzed as described above. The mass of the released peptide was 2471.20 (Fig. 1C), consistent with an acetylated N terminus. The next set of experiments was designed to determine whether growth of cells in media enriched with different dietary fatty acids could alter the profile of fatty acids acylated to Fyn. COS-1 cells transfected with (C3,6S)Fyn16TevEGFPsi6 were grown in media containing 5-cis-14:1 fatty acid, and the protein was purified, cleaved with TEV protease, and analyzed by mass spectrometry. As depicted in Fig. 1D, in addition to the peak of \(m/z = 2625.44\) corresponding to 14:0 modified Fyn, an additional peak of \(m/z = 2623.28\) was apparent, which likely represents Fyn modified with the 14:1 fatty acid. This conclusion is supported by the finding that incubation of cells in media containing 5-cis,8-cis-14:2 fatty acid resulted in the generation of a peak of \(m/z = 2621.17\) in addition to the 2625.39 myristoylated peak. The percent substitutions with 14:1 and 14:2, as calculated from the second isotopic peak in each of the experiments depicted in Fig. 1, D and E, respectively, are 43% for 14:1 and 58% for 14:2. Taken together, these data indicate that heterogeneous N-myristoylation of Src family kinases can occur and that the profile of attached fatty acids can be regulated by the abundance of various dietary fatty acids in the growth media.

We next attempted to exploit mass spectrometry to identify the fatty acids acylated to wild type Fyn. The TEV cleavage site was introduced after amino acid 16 of wild type Fyn, and a
Cells were lysed, immunoprecipitated with anti-Fyn antibody, incubated in media containing various iodo-fatty acid analogs. Labeled with [3H]arachidonate was visible after a 3-month exposure.

**Experimental Procedures.**

As depicted in Fig. 2, labeling of Fyn with [3H]oleate could be reversed by hydrophobicity of this peptide promotes sticking to the reversed phase columns that are used to purify the peptide prior to mass spectrometric analysis. A pilot experiment performed with a synthetic myristoylated, palmitoylated tetrapeptide revealed that this was indeed the case. Due to these technical limitations, we resorted to incubating cells with various radiolabeled fatty acids in order to identify potential heterogeneous S-acylation of Fyn.

**Src Family Kinases Can Be S-Acylated with Dietary Fatty Acids**—Previous studies (6) have reported that radiolabeled fatty acids other than palmitate were attached to platelet proteins by thioester linkage. However, the identity of the heterogeneously acylated proteins was not determined. We therefore tested whether Src family kinases could be S-acylated by dietary fatty acids. COS-1 cells expressing Fyn were grown in media supplemented with either tritiated palmitate, oleate, or arachidonate. Cells were lysed, immunoprecipitated with anti-Fyn antibody, and analyzed by SDS-PAGE and fluorography. As depicted in Fig. 2, labeling of Fyn with [3H]oleate could be visualized after a 10-day exposure of the gel to film, and Fyn labeled with [3H]arachidonate was visible after a 3-month exposure. Western blotting of the immunoprecipitated Fyn proteins with anti-Fyn antibody revealed that the total amount of Fyn protein was the same in each sample. These data suggest that unsaturated fatty acids can be incorporated into Src family kinases in vivo. It is also possible that some of the labeling may represent incorporation of fatty acid metabolites (see below).

**Incorporation of Iodinated Fatty Acid Analogs into Wild Type and Mutant Fyn**—The extremely long exposure times required for detection of the tritiated compounds prompted us to synthesize iodo-fatty acid analogs containing the high energy isotope 125I. We described previously (21) the synthesis of 13-iodotridecanoic acid (IC13:0) and 16-iodohexadecanoic acid (IC16:0), fatty acid analogs of myristate and palmitate containing iodine at the α carbon of the fatty acyl chain. As described under “Experimental Procedures,” we have now synthesized 18-iodooctadecenoic acid (IC18:1) and 18-iodooctadecanoic acid (IC18:0), iodo-fatty acids analogs of oleate and stearate, respectively. Each analog was radioiodinated by exchange with Na125I. COS-1 cells expressing wild type and mutant Fyn were incubated in media containing various iodo-fatty acid analogs. Cells were lysed, immunoprecipitated with anti-Fyn antibody, and analyzed by Western blotting and phosphorimaging. As depicted in Fig. 3, all of the analogs (IC13:0, IC16:0, IC18:0, and IC18:1) were incorporated into wild type Fyn. Mutation of glycine 2 to alanine prevented incorporation of all the fatty acid analogs (lanes 2, 5, 8, and 11), indicating that N-myristoylation is required for S-acylation. Previous studies have shown that cysteines 3 and/or 6 of Fyn are the sites for S-acylation with palmitate via a thioester linkage (8, 15, 16, 19). Mutation of both cysteines at position 3 and 6 did not affect incorporation of the myristate analog but abolished the incorporation of either iodinated palmitate (lane 6), oleate (lane 9), or stearate (lane 12). These results imply that cysteines 3 and 6 are potential sites for differential S-acylation.

**Incorporation of Iodo-Fatty Acid Analogs into Other Palmitoylated Proteins**—We next investigated whether differential S-acylation could occur on other cellular proteins known to be palmitoylated. Three proteins were chosen for further study. The Go subunit of the heterotrimeric G protein is both myristoylated and palmitoylated at an N-terminal Gly-Cys motif (22, 23). The neuronal protein GAP43 (neuromodulin) is palmitoylated at cysteines 3 and 4 (24). Ha-Ras is palmitoylated upstream of the C-terminal CAAX box (25). Chimeric proteins were generated by replacing the first 10 amino acids of wild type Fyn with the first 10 amino acids of Goα or GAP43 to generate Goα(10)-Fyn or GAP43(10)-Fyn. The G2A,C85F,Fyn-Ha-Ras construct was prepared by fusing the Ha-Ras tail to the C terminus of a Fyn mutant lacking the N-terminal fatty acylation sites. Palmitate incorporation into these chimeric constructs has been described previously (15, 16, 18). In order to test for differential S-acylation, cells expressing the Fyn chimeras were incubated with either iodinated palmitate, oleate, or stearate. Cells were then lysed, immunoprecipitated with anti-Fyn antibody, and analyzed by Western blotting and phosphorimaging. As shown in Fig. 4, modification of Fyn and the Fyn chimeras by oleate (lanes 5–8) and stearate (lanes 9–12) analogs as well as the palmitate (lanes 1–4) analog was observed. In addition, incorporation of these radioiodinated analogs into full-length G12V-Ha-Ras was detected (Fig. 4B). These data suggest that other palmitoylated proteins can also be modified by dietary fatty acids.

To examine the chemical nature of the linkage between these dietary fatty acids and proteins, samples of the GAP43(10)-Fyn protein labeled with either iodinated palmitate, oleate, or stearate were treated with hydroxylamine. As depicted Fig. 4C, the radiolabel was almost entirely removed after hydroxylamine treatment, indicating that fatty acid analogs of palmitate, oleate, and stearate form a thioester linkage with the proteins.

**Radiolabeled Fatty Acid Analysis by TLC**—The next set of...
experiments was designed to identify the fatty acids bound to proteins by thioester linkage. Fyn proteins labeled with either iodinated palmitate, oleate, or stearate were treated with sodium hydroxide to hydrolyze the bound, labeled fatty acids. The released fatty acids were then analyzed by reverse phase thin layer chromatography. As depicted in Fig. 5, when cells were incubated with the palmitate analog (IC16:0), 90% of the hydrolyzed fatty acids from Fyn (lane 4) migrated the same distance as the standard IC16:0 (lane 1). An additional spot was also observed upon longer exposure (10% of the total), which may represent myristate or another metabolic intermediate of the palmitate analog. The oleate analog (IC18:1) migrated essentially the same distance as the palmitate analog (IC16:0) on reverse phase TLC (compare lane 1 to lane 2) since their polarities are quite similar. When cells were incubated with oleate analog (IC18:1), ~55% of the hydrolyzed fatty acids (lane 5) migrated the same distance as the standard IC18:1 (lane 2). Surprisingly, about 45% of hydrolyzed fatty acids migrated to the same distance as the metabolites of IC16:0 (lane 5). The IC18:1 metabolites are likely to be IC16:1. Furthermore, alkali hydrolysis of Fyn labeled with the stearate analog (IC18:0) yielded ~33% IC18:0, 65% IC16:0, and 2% of possible IC14:0 (compare lane 6 to lanes 1 and 3). These results indicate that

Fyn Acylated with Unsaturated Fatty Acids Is Displaced from Rafts—Since the above experiments demonstrated that Fyn can be modified by dietary fatty acids, we next examined whether these modifications influence membrane localization and thereby affect Fyn function. Previous studies established that membrane targeting of Fyn is dependent upon dual fatty acylation. When COS-1 cells were labeled with either iodinated palmitate, oleate, or stearate, followed by fractionation into cytosolic (S100) and membrane (P100) fractions, both labeled Fyn and total Fyn proteins were localized almost entirely in the membrane fraction (Fig. 6A).

The ability of these differentially fatty acylated proteins to partition into rafts was then determined. Cells were incubated with either iodinated palmitate, oleate, or stearate, extracted with 0.5% Triton X-100, and the lysates were fractionated on Optiprep density gradients. Raft containing detergent-resistant microdomains were collected in the top fraction, and the Triton-soluble proteins were collected in the bottom fraction. Fractions were immunoprecipitated with anti-Fyn antibody and then analyzed by Western blotting with anti-Fyn antibody and phosphorimaging. As shown in Fig. 6, B and C, in cells treated with the palmitate analog (IC16:0), 73.7 ± 3% of the labeled Fyn was localized in the raft fraction. A similar result was obtained when cells were incubated with the stearate analog (IC18:0), with 72.1 ± 3% of labeled Fyn localized in rafts. However, incubation with the oleate analog (IC18:1) reduced the ability of labeled Fyn to localize to detergent-resistant microdomains, with only 41.1 ± 5% found in rafts. The amount of total Fyn protein in rafts was similar for cells treated with either palmitate, oleate, or stearate analogs with 34.1 ± 2, 27.6 ± 2, or 29.2 ± 4% in rafts, respectively. This indicates that the population of radiochemically labeled Fyn is a small percentage of the total Fyn population. There was no change in raft localization for the marker protein caveolin, indicating that raft structure was not altered by incubation with the various radiolabeled fatty acids. We also examined the
Effect of differential N-myristoylation on raft localization. COS-1 cells expressing wild type Fyn were incubated with myristic acid (14:0) or 5-cis,8-cis-tetradeacadienoic acid (C14:2) for 12 h, and the cells were Triton X-100 extracted and fractionated in Optiprep flotation gradients as described under “Experimental Procedures.” Fractions were collected from the top to the bottom of the gradient. Fractions were either trichloroacetic acid-precipitated or immunoprecipitated (IP) with anti-Fyn antibody and analyzed by Western blotting (WB) and phosphorimaging. A, distribution of Fyn in S100 (S) and P100 (P) fractions. B, distribution of Fyn in Optiprep flotation gradient. C, quantitation of total Fyn and labeled Fyn in rafts.


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Effect of PUFAs on Raft Localization of Fyn and LAT—In order to determine the consequences of fatty acylation with unsaturated fatty acids on signal transduction, we extended our studies into Jurkat T cells. The localization of Fyn and LAT, a palmitoylated raft localized protein, was determined. Jurkat T cells were incubated with either stearic acid, arachidonic acid (20:4), or eicosapentaenoic acid (20:5) and then stimulated with OKT3 antibody. Cell lysates were analyzed by Western blotting for pErk and total MAP kinase. As depicted in Fig. 8, PUFAs (20:4 and 20:5) inhibited activation of MAP kinase by ~70%, whereas saturated fatty acids (18:0) had virtually no effect. The levels of total MAP kinase remained unchanged.

DISCUSSION

**Heterogeneous N-Myristoylation of Src Family Kinases**—In this work, we have used the Src family kinase Fyn as a model to demonstrate directly that heterogeneous fatty acylation can occur on myristoylated as well as myristoylated, palmitoylated proteins. Previous studies have identified 12:0, 14:0, 14:1, and 14:2 fatty acids acylated via amide linkage to the N-terminal Gly of proteins such as recoverin, transducin, and catalytic subunit of protein kinase A (1–4). However, heterogeneous
Fatty Acylation, Raft Localization, and Src Family Kinases

N-myristoylation was believed to be restricted to the retina, as proteins isolated from brain, heart, and liver contained only amide-linked 14:0 myristate. Here, we exploited high resolution mass spectrometric methods to identify directly the fatty acid(s) attached to the N terminus of Fyn. Insertion of a unique protease cleavage site into the Fyn sequence facilitated these analyses, by allowing us to generate a unique N-terminal peptide whose molecular mass could be directly measured. As depicted in Fig. 1, 14:0 myristate was the predominant modification at the N terminus of Fyn when COS-1 cells were maintained in standard growth media. However, when cells were incubated in media enriched in unsaturated myristate analogs, the N terminus of Fyn became modified with 14:1 and 14:2 fatty acids. These data indicate that heterogeneous N-myristoylation can occur outside of the retina and is readily apparent when cells are grown in media enriched in various dietary 14-carbon fatty acids.

**Heterogeneous S-Acylated of “Palmitoylated” Proteins—**

Palmitoylation of Fyn requires prior N-myristoylation. Attempts to analyze a myristoylated, palmitoylated Fyn peptide by mass spectrometry were unsuccessful due to the high hydrophobicity of the dually acylated peptide. We therefore utilized a radiolabeling approach in order to examine the ability of fatty acids other than palmitate to be S-acylated to Fyn. Incubation of COS-1 cells expressing wild type Fyn with 3H-fatty acids revealed that palmitate, oleate, and arachidonate could be attached to Fyn. However, extremely long exposure times were required to visualize the tritiated Fyn band on fluorograms. A novel feature of the current study is the synthesis and radiiodination of stearate and oleate analogs that were then used to document incorporation of these longer chain fatty acids into Fyn using PhosphorImager technology. These methods have previously been successfully used by our laboratory to study attachment of iodinated myristate and palmitate analogs to Src family kinases (15, 18, 19). Here we show that, like palmitate, attachment of stearate and oleate required N-myristoylation of Gly-2 of Fyn, and mutation of cysteines at positions 3 and 6 prevented radiolabel incorporation into Fyn. These data strongly suggest that Fyn can be S-acylated with stearate, oleate, and arachidonate as well as palmitate.

The use of dietary fatty acids as alternative substrates for palmitoylsacryltransferase is not unexpected. Studies of partially purified palmitoyltransferase revealed that longer chain fatty acyl CoAs, including stearoyl-CoA and arachidonyl-CoA, can compete with palmitoyl-CoA for incorporation into Fyn and Gaα (26, 27). Moreover, TLC analysis of the radiiodinated fatty acids released from Fyn by alkaline hydrolysis revealed the presence not only of palmitate, oleate, and stearate but also of additional spots. We presume that these additional spots represent metabolic intermediates that result from one or two cycles of β-oxidation of the iodo analogs. Identification of these other acylated species is currently in progress.

Previous studies have documented incorporation of stearate, oleate, and linoleate into specific proteins such as P-selectin, the asialo-glycoprotein receptor, and Ga subunits (28–30). Base hydrolysis of total protein from heart and liver revealed thioester-linked 14:0, 18:0, 18:1, and 18:2 fatty acids in addition to 16:0 palmitate (4). Here, we show that iodinated analogs of palmitate, oleate, and stearate can be S-acylated to the N-terminal sequences derived from Gaα and GAP43, as well as the C-terminal prenylated tail of Ha-Ras. Thus, it is likely that heterogeneous S-acylation is a common occurrence for “palmitoylated” proteins.

A newly emerging theme is that the nature of the fatty acid(s) attached to acylated proteins can be regulated by altering the pool of fatty acids supplied in the diet. This study has shown that mono- and polyunsaturated fatty acids supplied in the growth medium can be directly attached to both the N-myristoylation and palmitoylation sites within the N terminus of Fyn, i.e. Gly-2 and Cys-3 and/or -6, respectively. Studies of platelets revealed that the pool of fatty acids covalently bound to platelet proteins by thioester linkage can be altered by exogenously supplied fatty acids (6). As discussed below, alteration of the fatty acylation pattern has functional significance, in that raft localization and signal transduction efficiency is altered.

**Acylation of Fyn with Unsaturated Fatty Acids Results in Decreased Raft Association—**Lipids in rafts appear to be present in a liquid ordered phase and primarily contain saturated fatty acids (31, 32). Proteins that contain saturated fatty acids, such as Src family kinases, endothelial nitric-oxide synthase, and Ga, are enriched in rafts, and insertion of a dual saturated fatty acyl anchor into a liquid ordered lipid domain is thought to be energetically favorable (33). The presence of cis double bonds would be expected to result in exclusion of proteins acylated with unsaturated fatty acids from rafts, as it is more difficult to pack these bulky side chains into the ordered lipid domain of the raft. This supposition was verified by a recent in vitro study, in which Go, which was S-acylated with unsaturated fatty acids and reconstituted into artificial liposomes was shown to be relatively less resistant to Triton X-100 extraction when compared with Go acylated with palmitate (34). Here we establish this principle in vivo, by showing that raft partitioning of Fyn acylated with radiolabeled mono- or polyunsaturated fatty acids is significantly lower than that of Fyn acylated with saturated fatty acids. Taken together, the data in this paper directly establish that incorporation of dietary unsaturated fatty acids can change the membrane localization of Src family kinases in vivo, by altering the interaction between fatty acids and the membrane.

**Effect of PUFAs on Raft Localization and Signal Transduction in Jurkat T Cells—**Much attention has been focused on long chain PUFAs, especially those found in fish oils, as certain PUFAs have been reported to exert a protective effect on tumor development. Whereas the molecular mechanism of the PUFA effect on tumorigenesis is far from understood, there is abundant evidence to indicate that PUFA treatment inhibits activation of T cells mediated by the activated T cell receptor (TCR). Incubation of Jurkat T cells with the PUFAs arachidonic acid (20:4) or eicosapentaenoic acid (20:5) attenuates the Ca2+ response (14) and inhibits MAP kinase activation (Fig. 8) by the activated TCR. We had shown previously that, in cells supplemented with arachidonate, Fyn still fractionates with the P100 membrane fraction, consistent with the notion that arachidonate replaces palmitate in the covalent modification of Fyn (15). The evidence presented in this paper strongly argues that the PUFA effect is due to S-acylation of PUFAs to the Src
family kinases Lck and Fyn, as well as LAT, which results in decreased raft localization of these critical signaling proteins and reduced TCR-mediated signaling. It is also possible, as has been suggested by other investigators (14), that PUFA treatment alters the lipid/fatty acid composition of the inner leaflet of the plasma membrane and thereby disrupts raft structure. However, at least in COS-1 cells, no change in the localization of caveolin-1, a marker for raft-like microdomains, was noted in PUFA-treated cells (15). Although there is a good correlation between the loss of Fyn and LAT from rafts and reduced signaling, it is still possible that other signaling molecules are also affected by incubation of cells with PUFAs.

In conclusion, the data presented in this paper demonstrate that Src family kinases can be fatty acylated with dietary fatty acids other than myristate and palmitate and that differential fatty acylation with unsaturated fatty acids results in the displacement of Src family kinases from membrane rafts. The relocalization of these proteins affects protein-protein and protein-lipid interactions and thereby attenuates downstream signaling events. Finally, the inhibition of lymphocyte proliferation by PUFAs suggests that dietary lipid manipulation could potentially be useful for treatment of various inflammatory diseases.

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