The N-terminal SH4 Region of the Src Family Kinase Fyn Is Modified by Methylation and Heterogeneous Fatty Acylation

ROLE IN MEMBRANE TARGETING, CELL ADHESION, AND SPREADING*

Nearly all Src family kinases (SFKs)* contain an N-terminal Met-Gly-Cys consensus sequence that promotes dual fatty acylation with myristate and palmitate. After removal of Met, myristate is co-translationally attached to the N-terminal Gly-2 via amide linkage, whereas palmitoylation of Cys-3 occurs post-translationally via a thioester linkage (1, 2). To date, studies of acylation of SFKs have been based solely on incorporation of radioactive myristate or palmitate. No study has directly examined the nature of the attached fatty acids in vivo. We recently exploited mass spectrometry to identify S-acylated species of the palmitoylated protein neuromodulin (GAP43) (3). Here we extend this approach to study the modifications on dually myristoylated and palmitoylated proteins, such as SFKs. Our results indicate that the SFK Fyn is efficiently myristoylated and that some of the myristoylated Fyn is also heterogeneous S-acylated with different dietary fatty acids.

During the course of this study, we identified an additional modification on Fyn: trimethylation of a lysine residue(s). Protein methylation involves transfer of a methyl group from S-adenosylmethionine to arginine, lysine, histidine, or carboxyl groups on proteins. Recently, protein methylation has emerged as an intensively studied regulatory modification of proteins. For example, carboxyl methylation of Ras is important for plasma membrane localization of Ras proteins (4). Most heterogeneous nuclear ribonucleoproteins contain multiple arginine-glycine repeats and are methylated by protein arginine methyltransferases. Methylation of heterogeneous nuclear ribonucleoproteins is essential for proper localization and function in RNA transport (5–7). A number of proteins have been shown to be methylated at lysine residues, including histones, ribulose-1,5-bisphosphate carboxylase/oxygenase (8), calmodulin (9), and cytochrome c (10). Methylation of Lys-9 in histone H3 regulates chromatin structure and gene silencing (11–14), whereas methylation of Lys-4 in histone H3 antagonizes the gene silencing effect of the Lys-9 methylation (15, 16).

Most of the SFKs contain multiple lysine residues near their N termini. In the context of Src, these lysines are part of a basic patch that promotes electrostatic interactions with acidic phospholipids in the membrane bilayer (17, 18). However, point mutations of individual lysines have revealed an additional role in myristoylation. For example, mutation of Lys-7 in v-Src greatly reduces myristoylation, membrane association, and transforming activity (19). Studies from our laboratory have shown that Lys-7 and Lys-9 of Fyn reduced myristoylation and membrane association of Fyn (20). These defects could be rescued by co-expression of exogenous N-myristoyltransferase (NMT). In this study, we demonstrate that the Src family kinase Fyn is methylated at lysine residues within its N-terminal region. Myristoylation and palmitoylation of Fyn are required for methylation. In contrast, treatment of cells with a general methylation inhibitor did not inhibit myristoylation and palmitoylation, suggesting that methylation of...
Methylation of Src Family Kinase Fyn

Fyn occurs after myristoylation and palmitoylation. Furthermore, we show that expression of EGFP fusion proteins containing wild type Fyn, but not the non-methylated mutants, promotes cell adhesion and spreading, suggesting that methylation of Fyn is essential for its proper localization and function.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were purchased as indicated: Tev protease (Invitrogen); Ni-NTA-agarose (Qiagen, Valencia, CA); defatted bovine serum albumin (Sigma); Na<sup>125</sup>I, l-[<sup>3</sup>H]methylthioadenosine, l-[<sup>3</sup>H]methyl-H<sub>4</sub>methionine, and S-adenosyl-l-[<sup>3</sup>H]methionine (PerkinElmer Life sciences); 5'-deoxy-S'-methylthiouridine (MTA), and fibronectin (Calbiochem); and pEGFP-N1 (Clontech). Synthesis and radioiodination of the IC13 (13-iodotridecanoic acid) and IC16 (16-iodohexadecanoic acid) fatty acid analogs were performed as described previously (2). Mouse anti-Fyn antibody was obtained from BD Biosciences. Mouse anti-GFP antibody was purchased from Roche Applied Science. Texas Red-X phalloidin and Prolong mounting medium were purchased from Molecular Probes. The rabbit polyclonal antisera to Fyn used for immunoprecipitation was described previously (21). Western blots were detected with ECL reagents (Amersham Biosciences).

**Plasmids**—The Fyn<sub>16TevEGFPHis6</sub> chimeric construct was prepared by fusing the sequences encoding the first 16 amino acids of Fyn to the 5′-terminus of Fyn<sub>16Tev</sub> to facilitate protein purification.

7-amino-acid (Glu-Asn-Leu-Tyr-Phe-Gln-Gly) cleavage site for Tev protease was generated by fusing the sequences encoding the first 16 amino acids of Fyn to the C-terminus of Fyn<sub>16Tev</sub> to facilitate protein purification.

Full-length wild type Fyn and G2A, C3S,C6S, K7A, K9A, or K7A,K9A mutant Fyn were prepared as described previously (2, 20). To prepare full-length FynEGFP fusion constructs, PCR products of wild type Fyn and G2A, C3S,C6S, K7A, K9A, or K7A,K9A mutants with the stop codon of Fyn deleted were inserted in-frame into EcoRI/BamHI-cut pEGFP-N1. All mutations were verified by DNA sequencing.

**Purification of Fyn Chimeric Proteins**—Ten plates of COS-1 cells, maintained in DMEM medium supplemented with 10% FBS, were co-transfected with human NMT and Fyn<sub>16TevEGFPHis6</sub> cDNAs at a 3:1 ratio using LipofectAMINE 2000 (Invitrogen). Two days after transfection, the cells were lysed in hypotonic buffer containing 20 mM Tris (pH 7.4), 0.2 mM MgCl<sub>2</sub>, and protease inhibitors. Pellets were harvested by centrifugation and centrifuged with RIPA buffer containing 20 mM Tris (pH 7.4), 500 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS (buffer A). After clarification by centrifugation, the detergent-insoluble pellets were further extracted with buffer A containing 8 M urea (buffer B). After centrifugation at 100,000 × g for 15 min, the supernatant was collected, diluted 4-fold with 20 mM Tris (pH 7.4), and loaded onto a 1-ml Ni-NTA column equilibrated previously with 4-fold diluted buffer B. After extensive washing, the column was eluted with 20 mM Tris (pH 7.4), 200 mM imidazole, 8 M urea (buffer B). For post-source decay experiments, data were acquired over 12 equal segments of m/z values between 0 and 1120, with reflectron voltages calculated at the New York University Protein Analysis Facility. The percentage of cells attached to the dishes was then determined at the New York University Protein Analysis Facility. The percentage of cells attached to the dishes with equal cell density was labeled with 10 μCi/ml IC13 or IC16 in DMEM containing 2% dialyzed FBS and 0.5% defatted bovine serum albumin in the presence or absence of MTA. Cells were lysed in RIPA buffer. Rabbit anti-Fyn antibody was added to cell lysates, and immunocomplexes were analyzed by SDS-PAGE followed by phosphorimaging (Amersham Biosciences) or Western blotting with mouse anti-Fyn antibody. Quantitation of the data from PhosphorImager screens was performed with ImageQuant software (Amersham Biosciences). Western blots were exposed to BioMax MR film (Eastman Kodak Co.), scanned with an Epson scanner, and quantitated with MacBas software.

**RESULTS AND DISCUSSION**

**N-terminal Fatty Acylation of the Src Family Kinase Fyn**—Mass spectrometry was used to examine the molecular identity of the fatty acid(s) attached to the N-terminal region of the Src

![Fig. 1](image-url)
family kinase Fyn. To minimize interference from internal proteolytic peptides, a chimeric GFP construct, Fyn\textsubscript{16TevEGFP}, was designed by inserting a specific Tev protease cleavage site after the N-terminal 16 residues of Fyn. A hexa-histidine tag was added at the C terminus to facilitate protein purification (Fig. 1A). Fyn\textsubscript{16TevEGFPHis\textsubscript{6}} was coexpressed in COS-1 cells with human NMT to ensure that the amount of endogenous NMT would not be limiting. Cells were then lysed in hypotonic buffer. As expected, nearly all of Fyn\textsubscript{16TevEGFPHis\textsubscript{6}} protein was present in the pellet. Interestingly, addition of RIPA buffer plus 8 M urea was required to completely solubilize the protein. When the pellet was extracted with RIPA buffer (pH 7.4), only 10% of the total Fyn\textsubscript{16TevEGFPHis\textsubscript{6}} protein was solubilized as compared with the amount extracted with RIPA plus urea (Fig. 1B). The solubilized Fyn\textsubscript{16TevEGFPHis\textsubscript{6}} protein was purified through a Ni-NTA-agarose affinity column with an 80% yield (Fig. 1B). The purified protein was concentrated and digested with Tev protease. Approximately 90% of the protein was cleaved based on the mobility shift observed by Western blotting with anti-GFP antibody (Fig. 1C).

Analysis of the digestion mixture by MALDI-TOF mass spectrometry revealed that Fyn\textsubscript{16TevEGFPHis\textsubscript{6}} was efficiently myristoylated at the N-terminal glycine (Fig. 2). The calculated monoisotopic m/z of the protonated myristoylated Fyn N-terminal peptide with a disulfide bond (i.e. m/z of \( \text{M} + \text{H}^+ \)) is 2655.34. A peak at m/z 2655.31 was observed, consistent with this peptide with an intramolecular disulfide between Cys-3 and Cys-6 (Fig. 2). The identity of the myristoylated peptide was confirmed by MALDI-TOF post-source decay sequence analysis (data not shown). The calculated monoisotopic mass of a protonated peptide that is both myristoylated and palmitoylated is 2895.58. As depicted in Fig. 2, a peak consistent with
this peptide at $m/z = 2895.55$ was clearly observed. Interestingly, a peptide of $m/z = 2893.58$ was also apparent, which is exactly 2 Da less than the palmitoylated peptide, suggesting that Fyn16TevEGFPHis$_6$ was heterogeneously acylated by palmitoleate or palmitate. A peak at $m/z = 2923.55$ was also detected, which is 28 Da larger than the palmitoylated peptide, suggesting that a small percentage of myristoylated Fyn peptides was acylated by stearate instead of palmitate. Close examination of the spectrum revealed the presence of a peptide at $m/z$ 2921.52, consistent with the Fyn peptide modified by myristate and oleate. This agrees with our previous studies with radiolabeled fatty acids, which revealed that heterogeneous fatty acylation of Fyn (2) as well as GAP43 can occur (3). In addition, a small peak at $m/z = 3133.64$ was also detectable, which corresponds to a peptide modified by one myristate and two palmitates. No peaks corresponding to the calculated $m/z$ (2447.15) of unmodified Fyn N-terminal peptides were observed.

Fyn$_{16}$TevEGFPHis$_6$ and Wild Type Full-length Fyn Are Methylated at Internal Lysine Residues—Most of the Src family tyrosine kinases contain multiple lysine residues within their N-terminal regions. Close examination of the mass spectra of the acylated peptides of Fyn$_{16}$TevEGFPHis$_6$ revealed several peaks that were 43 Da larger than the myristoylated or dually acylated peptides. The 43-Da increase in mass is consistent with trimethylation of a lysine residue(s) on the peptide. The peak at $m/z = 2700.30$ corresponds to a myristoylated and trimethylated peptide, whereas the peak at $m/z = 2938.54$ corresponds to a peptide modified by myristoyl, palmitoyl, and trimethyl groups. A small peak at $m/z = 2743.37$ was also detected, which is 43 Da more than the singly trimethylated peptide at 2700.30, suggesting that at least two lysines within the Fyn N-terminal region are trimethylated.

We next performed an in vivo methylation assay to confirm Fyn methylation. This method is based on the fact that the methyl group donor $S$-adenosylmethionine is derived from free methionine in vivo. COS-1 cells expressing wild type or mutant full-length Fyn were incubated with $L$-[methyl-$^3$H]methionine in the presence of cycloheximide and chloramphenicol. Cell lysates were immunoprecipitated with anti-Fyn antibody followed by SDS-PAGE, Western blotting (WB) with anti-Fyn antibody, and fluorography. To minimize interference from the heavy chain of IgG, samples were treated with SDS-PAGE sample buffer lacking β-mercaptoethanol. As a result, the Fyn band migrated as a doublet. In B, to verify that protein synthesis was fully inhibited, cells were labeled with $L$-[35S]methionine in the absence or presence of cycloheximide and chloramphenicol. Whole cell lysates were directly examined by phosphorimaging or immunoprecipitated (IP) with anti-Fyn antibody followed by Western blotting and phosphorimaging.

![Image](https://via.placeholder.com/150)

**Fig. 3.** Methylation of full-length Fyn. In A, COS-1 cells expressing wild type or mutant full-length Fyn were labeled with $L$-[methyl-$^3$H]methionine in the presence of cycloheximide and chloramphenicol. Cell lysates were immunoprecipitated with anti-Fyn antibody followed by SDS-PAGE, Western blotting (WB) with anti-Fyn antibody, and fluorography. To minimize interference from the heavy chain of IgG, samples were treated with SDS-PAGE sample buffer lacking β-mercaptoethanol. As a result, the Fyn band migrated as a doublet. In B, to verify that protein synthesis was fully inhibited, cells were labeled with $L$-[35S]methionine in the absence or presence of cycloheximide and chloramphenicol. Whole cell lysates were directly examined by phosphorimaging or immunoprecipitated (IP) with anti-Fyn antibody followed by Western blotting and phosphorimaging.

**Fig. 4.** The methylation inhibitor MTA does not inhibit myristoylation or palmitoylation of full-length Fyn. COS-1 cells expressing full-length Fyn were labeled with $L$-[methyl-$^3$H]methionine, myristate analog (IC13), or palmitate analog (IC16) in the absence or presence of the methylation inhibitor MTA. Cell lysates were immunoprecipitated with anti-Fyn antibody and analyzed by SDS-PAGE followed by phosphorimaging, fluorography, or Western blotting (WB) with anti-Fyn antibody.
and Palmitoylated when Coexpressed with NMT were generated, and the amounts of [125I][IC13 and [35S]methionine incorporation of the labeled myristate ([125I][IC13) or palmitate ([35S]methionine) into each protein were compared. In the absence of exogenous NMT, (K7A,K9A)FynEGFP exhibited myristoylation levels that were 40–50% those of wild type FynEGFP. Co-expression of NMT dramatically increased myristoylation of (K7A,K9A)FynEGFP to 85% (±2%) that of wild type FynEGFP. Because myristoylation is required for palmitoylation of Fyn, we also assessed levels of [125I][IC16 incorporation. The (K7A,K9A)FynEGFP mutant, when co-expressed with NMT, exhibited IC16 incorporation levels that were 74% (±8%) of wild type FynEGFP. Despite the fact that (K7A,K9A)FynEGFP had nearly wild type levels of myristoylation and palmitoylation, membrane binding of this mutant was reduced as compared with that of wild type protein. In the presence of NMT, >95% of FynEGFP fractionated in the 100,000 × g P100 membrane pellet, whereas only 40% of the (K7A,K9A)FynEGFP mutant was in the P100 (Fig. 5A). Confocal imaging of cells expressing FynEGFP proteins revealed plasma membrane localization for both wild type and mutant constructs (Fig. 5B). However, there was also significant cytosolic staining evident in cells expressing K7A and K7A,K9A FynEGFP, consistent with the biochemical fractionation data. Taken together, these data suggest that methylation of lysines 7 and/or 9 is required, in addition to myristoylation and palmitoylation, for proper membrane targeting of FynEGFP.

Lysine Mutants of FynEGFP Are Defective in Cell Adhesion and Spreading—A cell adhesion assay was employed to explore the function of Fyn methylation. COS-1 cells were co-transfected with NMT and wild type or lysine mutants of FynEGFP. Transfected cells were trypsinized, counted, and replated on fibronectin-coated dishes for various times. The unattached cells were then removed, and the attached cells were harvested and counted. The percentage of green cells in the original cell suspension and after resuspension of the attached cells was determined by fluorescence-activated cell sorter and used to determine the total number of green cells in each population. The percentage of cells attached was calculated by dividing the number of green cells attached to the dishes by the number of green cells in the original cell suspension. As depicted in Fig. 6A, expression of wild type FynEGFP promoted cell adhesion as compared with the GFP control. Mutation of lysine 7 or double mutation on lysines 7 and 9 greatly reduced the effect of FynEGFP-mediated cell adhesion, suggesting that methylation of FynEGFP is essential for its function in cell adhesion. It is not clear why the rates and extent of cell adhesion for the lysine mutants were lower than the control EGFP. One possibility is that these mutants function as dominant negative inhibitors of endogenous Fyn protein.

To further characterize the function of Fyn in cell spreading, cells expressing wild type and mutant FynEGFP were replated on coverslips for 1 h. Cells were fixed and permeabilized, and
Fig. 6. Wild type (Wt) FynEGFP, but not K7A or K7A,K9A mutants, promotes cell adhesion and spreading. In A, COS-1 cells expressing wild type or mutant FynEGFP were trypsinized and held in suspension for 1 h in DMEM medium containing 0.5% FBS. Cells were counted, and aliquots were plated on fibronectin-coated dishes. After the indicated times, the percentage of attached cells that expressed FynEGFP was calculated as described under “Experimental Procedures.” Results shown are the average of 3 independent experiments. In B, COS-1 cells expressing wild type or mutant FynEGFP were replated on coverslips for 1 h. Cells were fixed, permeabilized, and stained with Texas Red-phalloidin to reveal the morphology of the actin cytoskeleton. Nuclei were stained with Hoechst (4',6-diamidino-2-phenylindole). Cells were visualized by confocal microscopy.
the actin filaments were labeled with Texas Red-X phalloidin. Cells were visualized by fluorescence microscopy. As depicted in Fig. 6B, cells expressing GFP had few ruffles or other protrusions but exhibited stress fibers across the cell body. Cells expressing wild type FynEGFP had extensive lamellipodia and filopodia around the periphery of the cell but were nearly devoid of stress fibers. Furthermore, FynEGFP significantly overlapped with actin filaments at the leading edge of spreading cells, suggesting that Fyn is capable of rearranging the actin cytoskeleton. In contrast, the K7A and K7A,K9A mutant proteins were localized in the cytosol or intracellular membrane compartments. Cells expressing these mutants were significantly less able to extend membrane protrusions at the edge of spreading cells but were able to form stress fibers around the FynEGFP mutant proteins. These data suggest that lysines in the Fyn SH4 domain are essential for efficient plasma membrane targeting of Fyn as well as the function of Fyn in promoting formation of lamellipodia and filopodia.

In summary, we have identified methylation as yet another modification within the N-terminal SH4 domain of Fyn. Methylation of Fyn, suggesting that the Fyn methyltransferase is membrane-bound. It is interesting to compare the modifications in the N-terminal region of Fyn with those in the C-terminal region of Ras GTPases. Ras proteins are prenylated at the cysteine residue within the C-terminal CAAX box. The prenyl-CAAX motif is proteolytically cleaved, exposing the prenyl cysteine as the new C terminus. This modified cysteine is then recognized by a prenyltransferase that methylates the α-carboxyl group. Methylation of Ras is important for directing it to the plasma membrane, a finding analogous to our results with Fyn. However, it is likely that methylation may play a role beyond that of membrane targeting. Although the (K7A,K9A)FynEGFP mutant was myristoylated and palmitoylated to nearly wild type levels and exhibited significant spreading, cells expressing wild type FynEGFP had extensive lamellipodia and filopodia around the periphery of the cell but were nearly devoid of stress fibers. Furthermore, FynEGFP significantly overlapped with actin filaments at the leading edge of spreading cells, suggesting that Fyn is capable of rearranging the actin cytoskeleton.