Palmitoylation of p59^{fyn} Is Reversible and Sufficient for Plasma Membrane Association

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Members of the Src family of protein tyrosine kinases are localized to subspecialized regions of the plasma membrane. Herein we show that the N-terminal SH4 region of the Src family member p59^{fyn} (Fyn) is both necessary and sufficient for targeting of Fyn and heterologous proteins to the plasma membrane and detergent-insoluble subdomains. Attachment of the first 16 amino acids of Fyn to a normally cytosolic protein, β -galactosidase, resulted in distinct plasma membrane localization of the chimeric protein. Mutation of the palmitoylation site (cysteine-3) within Fyn16- β -galactosidase or wild-type Fyn abrogated plasma membrane localization, resulting in redistribution of the mutant proteins into intracellular membranes. Substitution of the SH4 motif within Fyn with heterologous sequences from other palmitoylated proteins (Gao and GAP43) revealed that the presence of palmitate is sufficient to direct plasma membrane localization independent of surrounding amino acid sequences and myristate. Palmitoylated Fyn chimeras were also enriched in the Triton X-100-resistant matrix, whereas nonpalmitoylated forms of these proteins were detected in the detergent-soluble fraction. The palmitate moiety on Fyn exhibited a half-life of 1.5–2 h. In contrast, the half-life of the polypeptide backbone was 8 h, indicating that palmitoylation is a reversible modification. These studies establish that the palmitoylated SH4 sequence of Fyn can be used to specifically target proteins to the plasma membrane in a reversible manner.

INTRODUCTION

The Src family consists of nine related protein tyrosine kinases that participate in signaling from cell surface receptors to the interior of the cell (reviewed in Cantley *et al.*, 1991; Bolen *et al.*, 1992). All members of the Src family are covalently modified by the 14-carbon fatty acid myristate. Myristate is cotranslationally attached to a glycine at position 2 of the protein through an amide linkage. Seven of the nine Src family proteins are also modified posttranslationally by the 16-carbon fatty acid palmitate. Palmitate is attached to cysteines near the N terminus of the protein through a

thioester linkage and represents a reversible modification of the protein. A palmitoyl acyltransferase that palmitoylates myristoylated Src family proteins has recently been characterized (Berthiaume and Resh, 1995).

Fatty acylation of the Src family members has been shown to influence their localization and interaction with other cellular components. One of the best studied examples is p59^{fyn} (Fyn), a 59-kDa member of the Src family modified by palmitate at cysteines 3 and 6 (Shenoy-Scaria *et al.*, 1993; Alland *et al.*, 1994). Palmitoylation of Fyn influences its ability to bind to cellular membranes (Alland *et al.*, 1994; Shenoy-Scaria *et al.*, 1994). In addition, palmitoylation of Fyn has been implicated in the ability of the protein to partition with a detergent-insoluble fraction formed by solubilizing cells with nonionic detergents. This fraction is a complex mixture consisting of at least three types of

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structures: detergent-insoluble glycosphingolipid microdomains enriched in glycosyl phosphatidyl inositol-anchored proteins (Rodgers *et al.*, 1994; Schnitzer *et al.*, 1995); caveolae, membrane invaginations containing the marker protein caveolin (Rothberg *et al.*, 1992); and cytoskeletal matrix (Lenk *et al.*, 1977; Ben-Ze'ev *et al.*, 1979; Cervera *et al.*, 1981). Interaction of Fyn with GPI-anchored proteins such as CD59 and decay-accelerating factor is dependent on palmitoylation of Fyn at cysteine 3 (Shenoy-Scaria *et al.*, 1993). It has been proposed that palmitoylation acts as a signal to direct Fyn to lipid-rich microdomains, facilitating its interaction with the GPI-anchored proteins that also localize to these domains. Palmitoylation of Fyn and of Hck, another Src family member, has also been implicated in localization of these tyrosine kinases to caveolae (Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995). Caveolin-containing microdomains are associated with, yet apparently distinct from, those plasma membrane fractions enriched in GPI-anchored proteins (Schnitzer *et al.*, 1995). In addition to localizing with GPI-anchored proteins, Fyn associates with several cytoskeletal proteins, including pp125^{FAK} and cortactin. These proteins are found at points of attachment between the cytoskeleton and the plasma membrane (Glenney and Zokas, 1989; Schaller *et al.*, 1992; Sartor and Robbins, 1993; Cobb *et al.*, 1994; Okamura and Resh, 1995).

The role of the N-terminal SH4 motif in directing fatty acylation and membrane targeting has not yet been fully defined. Herein we address three key issues regarding the biological function of dual fatty acylation. First, we demonstrate that the SH4 sequence is both necessary and sufficient for targeting of Fyn and heterologous proteins to the plasma membrane and the detergent-resistant matrix. This information can now be used to design Fyn SH4 fusion proteins to target normally cytosolic signaling molecules to specific subdomains of the plasma membrane. Second, we show that the Fyn SH4 sequence can be replaced with heterologous N-terminal sequences from other palmitoylated proteins. Palmitoylation and plasma membrane association of the Fyn chimeras can occur independently of myristate and the surrounding amino acid sequence. Third, we demonstrate that the turnover of the palmitate moiety on Fyn (1.5–2 h) is significantly shorter than the half-life of the polypeptide chain backbone (8 h), thereby establishing that palmitoylation of Fyn is a reversible modification *in vivo*. Nearly 50% of the Fyn protein in a cell was estimated to be nonpalmitoylated, and this population of Fyn molecules was localized to intracellular membranes and the detergent-soluble fraction. These studies demonstrate that palmitoylation of the SH4 sequence of Fyn specifically targets proteins to the plasma membrane in a reversible manner.

MATERIALS AND METHODS

Plasmid Construction

The plasmid pSP65/*NcoI*-fyn encodes wild-type (wt) human p59^{fyn} [a kind gift from P. Espino (Genzyme, Framingham, MA) and S. Courtneidge (EMBL, Heidelberg, Germany)]. The N-terminal pGEM-fyn mutants (C3, 6S fyn, C3S fyn, C6S fyn, and G2A fyn) were prepared as described (Alland *et al.*, 1994) and cloned into pSP65 by digesting the pGEM-fyn mutants with *NcoI* and *XhoI* and replacing the equivalent wt fyn fragment in pSP65/*NcoI*-fyn. The chimeric fyn mutants Gao-fyn and growth cone-associated protein (GAP)43-fyn were generated by polymerase chain reaction (PCR). Sense oligonucleotides were synthesized to encode either an *NcoI* site (Gao-fyn) or a *BamHI* site (GAP43-fyn) followed by the cDNA sequence corresponding to the first 10 amino acids of Gao or GAP43. The sense oligonucleotides and an antisense oligonucleotide corresponding to the SH3 domain of fyn were used along with pGEM-fyn as a template to generate mutant cDNAs by PCR. The PCR products were then digested with *NcoI* or *BamHI* and *BstXI* to generate a 76-bp fragment that was used to replace the corresponding fragment of wt fyn in pSP65. wt fyn and all of the fyn mutants in pSP65 were cloned into the retroviral vector pLJ (a kind gift from J. Schwartzbauer, Princeton University, Princeton, NJ) by digesting with *BamHI* and *Sall* and ligating the fyn fragments into *BamHI*-*Sall*-digested pLJ.

The fyn16- β -galactosidase (β gal) chimeras were generated by PCR. wt fyn and N-terminal fyn mutants in pGEM3Z containing the upstream region of *v-src* were amplified by using a sense primer containing an *XbaI* site corresponding to the upstream region of *v-src* and an antisense primer containing a *BamHI* site starting from the corresponding amino acid 16 in Fyn. The PCR products were then digested with *XbaI* and *BamHI* and ligated to *XbaI*-*BamHI*-digested p65 VALO vector (Picard and Yamamoto, 1987).

The fyn25-glutathione *S*-transferase (GST) chimera was generated by using PCR. First, GST was amplified using a sense primer containing a *SacI* site plus a 21-bp sequence from the 5' end of the GST cDNA (GATGAGCTCCCTATATACTAGGTTATTGG) and an antisense primer containing an *XhoI* site and 21 bp from the 3' end of the GST cDNA (CCGCTCGAGTTATTTGGAGGATGGTCCGCCACC). This was done to clone the N-terminal sequence of Fyn onto the N terminus of GST so that Fyn could be properly acylated with fatty acids *in vivo*. The PCR product was then digested with *SacI* and *XhoI* and cloned into a *SacI*-*XhoI*-digested pET11b-based plasmid coexpressing Fyn and hNMT (Peseckis and Resh, 1994). Digestion of the pET11b plasmid with *SacI* and *XhoI* removed all but the N-terminal 25-amino acid sequence of Fyn and replaced it with the GST sequence. The fyn25-GST DNA was then cloned into pCMV5 by digestion of both vectors with *BglII* and *XhoI*. All mutations were confirmed by sequencing.

Cell Culture and Transfections

NIH 3T3 cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% calf serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Transfection and G418 selection of NIH 3T3 cells was performed as reported previously (Cone and Mulligan, 1984). COS-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Transient transfection using DEAE-dextran was performed as reported previously (Alland *et al.*, 1994).

Antibodies

The rabbit polyclonal antiserum to Fyn used for immunoprecipitations was raised against a histidine-tagged fusion protein consisting of amino acids 1–148 of human Fyn, which includes the N terminus, the unique region, and SH3 domain of the protein. The rabbit polyclonal antiserum to Fyn used for immunofluorescence was

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal antibody to Fyn used for immunoblotting was purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibody to β gal was purchased from Promega (Madison, WI). Rabbit polyclonal antiserum to GST was purchased from Sigma (St. Louis, MO).

Preparation of Cell Lysates and Immunoblotting

Confluent 60-mm plates of cells were rinsed with ice-cold STE (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) and lysed in 1 ml of ice-cold lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 5 mM NaF, 1 mM sodium vanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Lysates were clarified at $100,000 \times g$ for 15 min at 4°C. The protein concentration in each lysate was measured with the Pierce micro-BCA protein assay (Rockford, IL). Equal amounts of protein from each lysate were immunoprecipitated with anti-Fyn antibody or anti- β gal antibody and protein A-agarose (Santa Cruz Biotechnology). Samples were washed three times with lysis buffer and analyzed by SDS-PAGE. Protein was transferred to Immobilon-P membrane (Millipore, Bedford, MA) at 480 mA for 1 h in 25 mM Tris, 192 mM glycine, and 20% methanol. The membrane was blocked in Tris-buffered saline (10 mM Tris, 0.9% NaCl, pH 7.4) containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h at room temperature. The primary antibody was diluted 1:500 (for Fyn blots) or 1:2000 (for β gal blots) in blocking buffer and the membrane was incubated overnight at 4°C in primary antibody solution. The membrane was washed extensively in Tris-buffered saline/0.1% Tween 20. The membrane was then incubated in a secondary antibody solution consisting of a 1:2000 dilution of horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) in blocking buffer. The membrane was washed as described and developed by using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Western blots were quantitated using the Millipore Bio-Image system.

Immunoprecipitations and In Vitro Kinase Assays

Cell lysates were prepared as described above. Equal amounts of protein from each lysate were incubated with 5 μ l of rabbit anti-Fyn antibody and 10 μ l of protein A-agarose for 2 h at 4°C. After immunoprecipitation, the protein A-agarose pellet was washed twice with ice-cold lysis buffer and once with ST buffer (10 mM Tris, pH 7.4, 150 mM NaCl). For the in vitro kinase assay, pellets were resuspended in 50 μ l of $1 \times$ Tris-Mg²⁺ and 17 μ Ci of [γ -³²P]ATP. Samples were incubated at room temperature for 15 min, and reactions were then quenched with 1 ml of ice-cold lysis buffer. Protein A beads were washed once with ice-cold lysis buffer, resuspended in $1 \times$ SDS sample buffer containing 0.1 M dithiothreitol, and loaded onto a 10% SDS polyacrylamide gel. Kinase assays were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Cell Labeling

The synthesis and radiolabeling of 16-[¹²⁵I]iodohexadecanoic acid (IC16) and 13-[¹²⁵I]tetradecanoic acid (IC13) has been described previously (Peseckis *et al.*, 1993). Each 60-mm plate of cells to be labeled was starved for 1 h with 1 ml of DMEM medium containing 2% dialyzed fetal bovine serum. The cells were then incubated for 5 h to overnight at 37°C with 50 μ Ci/ml IC16, 50 μ Ci/ml IC13, or 50 μ Ci/ml Tran³⁵S-label (ICN, Irvine, CA). Cells were washed twice with ice-cold STE and lysed in 1 ml of lysis buffer, and lysates were clarified at $100,000 \times g$ for 15 min at 4°C. Lysates were immunoprecipitated with rabbit anti-Fyn antibody and protein A-agarose. Immunoprecipitates were washed three times with ice-cold lysis buffer then resuspended in 20 μ l of $1 \times$ SDS sample buffer containing 20 mM dithiothreitol and subjected to SDS-PAGE on a 10% gel. IC16 gels were dried between cellophane sheets and exposed at

–80°C with two intensifying screens and [³⁵S]methionine gels were treated with 1 M salicylic acid and dried.

For pulse-chase experiments after cell labeling, cells were washed with DMEM and incubated with DMEM/2% dialyzed fetal bovine serum containing 5 mM methionine, 800 μ M unlabeled myristic acid plus 1% bovine serum albumin, or 200 μ M unlabeled palmitic acid plus 1% bovine serum albumin. These concentrations of methionine, myristic acid, and palmitic acid represent a 10-fold excess over the amounts used in the cell labelings. At various times after addition of chase medium, cells were lysed and processed as described above. Gels were quantitated by PhosphorImager analysis.

Membrane Fractionation

Cellular fractionation was performed as described (Alland *et al.*, 1994). Briefly, confluent 60-mm plates of cells were rinsed with ice-cold STE, scraped off the plate with a rubber policeman, and pelleted at $1500 \times g$ for 5 min. Cells were homogenized in 640 μ l of hypotonic buffer (10 mM Tris, pH 7.4, 0.2 mM MgCl₂, 1 mM sodium vanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF) with 30 strokes in a tight-fitting Dounce homogenizer. Homogenates were adjusted to 0.25 M sucrose and 1 mM EDTA and centrifuged at $100,000 \times g$ for 45 min. The resultant supernatants (S100) were brought to 1 ml in $5 \times$ lysis buffer. The pellets (P100) were resuspended in 1 ml of $1 \times$ lysis buffer and clarified at $100,000 \times g$ for 15 min. The S100 and P100 fractions were analyzed by immunoprecipitation and Western blotting as described above. For Fyn25-GST-expressing cells, the cells were first labeled with 50 μ Ci/ml [³⁵S]methionine and then fractionated. The Fyn25-GST protein was precipitated with 10 μ l of glutathione-agarose beads (Santa Cruz Biotechnology) and analyzed by SDS-PAGE and autoradiography.

Immunofluorescence Microscopy

Cells expressing the appropriate Fyn mutants were seeded onto 25-mm glass coverslips 1 d prior to the experiment. All subsequent steps were carried out at room temperature. The cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS as described (Silverman and Resh, 1992). Coverslips were incubated for 45 min with a primary antibody solution containing 2.5 μ g/ml of rabbit anti-Fyn antibody, 6 μ g/ml mouse anti- β gal antibody, or a 1:200 dilution of rabbit anti-GST antibody in PBS/10% calf serum. This was followed by a 30-min incubation with a secondary antibody solution containing a 1:75 dilution of fluorescein-conjugated goat anti-rabbit IgG or anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS/10% calf serum. In control experiments, primary antibody was first incubated overnight at 4°C with a 100-fold excess of the antigenic peptide against which it was raised (Santa Cruz Biotechnology) in PBS/10% calf serum. Coverslips were mounted in 90% glycerol, 1% phenylenediamine in PBS. Cells were observed with a 63 $\times$ oil-immersion lens on a Zeiss Axiophot microscope and photographed with Kodak Tri-X pan 400 film. Fyn25-GST-expressing cells were observed with a 100 $\times$ oil immersion lens on an Olympus Vanox microscope and photographed with Kodak Tri-X pan 100 film.

Triton X-100 Fractionation

Separation of cells into detergent-soluble and -resistant fractions was performed as described previously (Okamura and Resh, 1994). Briefly, confluent 60-mm plates of cells were washed with ice-cold STE and then incubated for 3 min at 4°C with 0.5 ml of Csk buffer [10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 M sucrose, 1% Triton X-100, 1 mM sodium vanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF] and then for 1 min with an additional 0.5 ml of Csk buffer. This fraction (S) is termed the detergent-soluble fraction. The remaining detergent-resistant matrix (R) on the dish was incubated

Table 1. Characteristics of Fyn mutants and chimeras

Construct	Amino acid ^a									Myristate ^b	Palmitate ^c	% Membrane bound ^d
	2	3	4	5	6	7	8	9	10			
wt Fyn	G	C	V	Q	C	K	D	K	E	+	++	88 ± 7
C3,6S Fyn	G	<u>S</u>	V	Q	<u>S</u>	K	D	K	E	+	—	63 ± 4
C3S Fyn	G	<u>S</u>	V	Q	C	K	D	K	E	+	—/+	67 ± 12
C6S Fyn	G	C	V	Q	<u>S</u>	K	D	K	E	+	+	90 ± 8
G2A Fyn	<u>A</u>	C	V	Q	C	K	D	K	E	—	—	32 ± 10
G _{ao} -Fyn	G	C	<u>T</u>	<u>L</u>	S	A	E	D	<u>K</u>	+	+	98 ± 1
GAP43-Fyn	<u>L</u>	C	<u>C</u>	<u>M</u>	<u>R</u>	<u>R</u>	<u>T</u>	K	<u>Q</u>	—	++	99 ± 1

^aSequences of the first 10 amino acids of each Fyn construct are shown with mutant amino acids underlined. The first 10 amino acids of wt Fyn have been replaced with those of G_{ao} and GAP43 in G_{ao}-Fyn and GAP43 Fyn, respectively.

^bResults of labeling NIH 3T3 cells with the iodinated myristic acid analogue IC13.

^cResults of labeling NIH 3T3 cells with the iodinated palmitic acid analogue IC16.

^dValues were obtained by quantitating Western blots from four separate experiments for each construct except G_{ao}-Fyn and GAP43-Fyn, which were acquired from two experiments. The first number represents the percentage in the P100 and the second number represents the SD from each set of experiments.

with 1 ml of ice-cold lysis buffer for 10 min and scraped off the dish with a rubber policeman. Fractions were clarified at 100,000 × g for 15 min at 4°C. The soluble fraction was corrected for detergent concentration with 5× lysis buffer. Both fractions were immunoprecipitated with anti-Fyn antibody and protein A-agarose and analyzed by SDS-PAGE followed by immunoblotting or autoradiography. For Fyn25-GST, cells were processed as described for the membrane fractionation.

RESULTS

Palmitoylation of Fyn Is Necessary for Plasma Membrane Localization

Previous studies by our laboratory and others have established that mutation of cysteine-3 reduces the extent of palmitoylation and membrane binding of Fyn (Alland *et al.*, 1994). To further define the effect of fatty acylation on the specific subcellular localization of Fyn, stable cell lines were created by infecting NIH 3T3 cells with recombinant retroviruses encoding wt and various mutant forms of Fyn (Table 1). The putative sites of palmitoylation were mutated individually (C3S and C6S) or together (C3,6S) by replacing cysteine with serine. Likewise, the site of attachment of myristate, glycine-2, was mutated to alanine to generate a nonmyristoylated protein. Quantitation of immunoblots of NIH 3T3 cell lysates indicated that the level of overexpression of wt Fyn was approximately 10 times higher than endogenous Fyn. All of the cell lines produced comparable levels of Fyn protein and tyrosine kinase activity (our unpublished results). Fatty acylation patterns were determined by radiolabeling with radioiodinated fatty acid analogues of palmitate (IC16) or myristate (IC13; Peseckis *et al.*, 1993) and are summarized in Table 1. In agreement with previously reported results in other cell types, cysteine-3 appears to be the main site of palmitoylation within Fyn.

The subcellular localization of each Fyn construct was next examined by fractionating cells into cytosolic (S100) and membrane (P100) fractions. Each fraction was analyzed by immunoblotting with anti-Fyn antibodies and quantitated by densitometry. wt Fyn was primarily present in the particulate fraction (88%, Table 1), whereas the distribution of nonpalmitoylated C3,6S Fyn was increased in the soluble fraction (37% soluble). C3S Fyn closely resembled C3,6S Fyn in that it was 33% soluble, and C6S Fyn mimicked wt Fyn and was 90% membrane associated. A greater effect was observed with G2A Fyn, which is neither myristoylated nor palmitoylated. Approximately 68% of this protein was present in the soluble fraction. These results indicate that fatty acylation of Fyn is important for its ability to associate with cellular membranes. The presence of palmitate has a slight effect on membrane association, whereas the presence of both palmitate and myristate greatly affect the ability of Fyn to bind to cellular membranes. In addition these results strengthen the conclusion that the cysteine at position 3 plays a greater role in the membrane localization of Fyn than the cysteine at position 6. It is important to note that the particulate fraction in this assay contains total cell membranes, including plasma membrane and intracellular membranes. Thus, changes in Fyn distribution among various cell membranes will not be detected, prompting us to employ additional cytological techniques.

To investigate the intracellular localization of Fyn more precisely, immunofluorescence of NIH 3T3 cells expressing wt Fyn and various Fyn mutants was undertaken. Cells were fixed, permeabilized, and incubated with rabbit anti-Fyn antibodies. An anti-rabbit antibody conjugated to fluorescein was used to visu-

alize Fyn staining. Figure 1A shows that wt Fyn was primarily present in both plasma membrane and intracellular membranes, as illustrated by bright staining near the perimeter of the cell and punctate intracellular staining. To demonstrate the specificity of Fyn staining, the primary antibody was preincubated with the peptide against which it was raised and then incubated with cells expressing wt Fyn. As illustrated in Figure 1B, antibody incubated with blocking peptide showed a low level of staining around the nucleus, indicating that some of the perinuclear staining was nonspecific. However, preincubation of the Fyn antibody with blocking peptide ablated membrane staining in NIH 3T3 cells expressing wt Fyn, indicating that the plasma membrane staining in Figure 1A was specific for Fyn. The parent NIH 3T3 cells were also stained with Fyn antibodies and showed very little staining (Figure 1H). In contrast to wt Fyn, nonpalmitoylated C3,6S Fyn showed almost no staining in the plasma membrane but instead exhibited a diffuse pattern of staining of intracellular membranes (Figure 1C). The nonacylated G2A Fyn also showed diffuse cytosolic staining (Figure 1D). Interestingly, C3S Fyn showed a low level of plasma membrane staining in addition to intracellular membrane staining (Figure 1E). This correlated well with results from Table 1, in that C3S Fyn had a low level of IC16 labeling. C6S showed the same pattern of staining as wt Fyn in that it was localized to both plasma membranes and intracellular membranes (Figure 1F). These results strengthen the observation that fatty acylation of Fyn is important for specifying the localization of the protein within the cell.

Palmitoylation of Heterologous Sequences Can Direct Proper Localization of Fyn

All of the mutations of Fyn examined thus far abolish myristoylation and/or palmitoylation of Fyn and result in an inability of the protein to target properly in the cell. In addition these mutations all occur within the context of wt Fyn. To separate the role of the surrounding amino acid sequence of the protein from the requirement for fatty acylation, two chimeric proteins were constructed. The amino acid sequences and characteristics of these proteins are listed in Table 1. Note that both chimeric proteins have very different N-terminal sequences than wt Fyn except for the specific amino acids that are acylated. Gao-Fyn contains the first 10 amino acids of the α subunit of the heterotrimeric G_o-protein instead of wt Fyn. This protein contains glycine at position 2 and cysteine at position 3 and is both myristoylated and palmitoylated (Linder *et al.*, 1993; Parenti *et al.*, 1993). In GAP43-Fyn, the first 10 amino acids of the neuronal protein GAP43 (also known as neuromodulin; Liu *et al.*, 1993) replace those of wt Fyn. GAP43 contains cysteines at positions 3 and

4 and is palmitoylated apparently at both cysteines. Since GAP43 does not have a glycine at position 2, it is not myristoylated. This chimeric protein therefore allowed us to separate the role of palmitoylation from myristoylation in the subcellular localization of the protein.

Stable NIH 3T3 cell lines were created for Gao-Fyn and GAP43-Fyn as described for the other Fyn mutants and assayed for expression of the chimeric proteins. Both Gao-Fyn and GAP43-Fyn were expressed in NIH 3T3 cells, although not to the same levels as wt Fyn (Figure 2A). Quantitation of immunoblots revealed that expression levels of Gao-Fyn and GAP43-Fyn were approximately 50% and 40% of wt Fyn, respectively. The autokinase activity of these chimeric proteins was examined in immunoprecipitates (Figure 2B). Comparison of protein levels versus immune complex kinase activity indicated that Gao-Fyn was approximately five times less active than wt Fyn and GAP43-Fyn was about six times less active than wt Fyn. To determine whether the decreased kinase activity was an intrinsic property of the chimeric Fyn proteins, we examined the autokinase activity of Fyn proteins expressed by *in vitro* translation. The specific activity of Gao-Fyn and GAP43-Fyn was twofold lower than that of wt Fyn produced *in vitro* in rabbit reticulocyte lysates. These results imply that replacement of the N terminus of Fyn has a deleterious effect on the kinase activity of the protein.

The palmitoylation state of the Fyn chimeras was also examined by labeling with the iodinated palmitate analogue IC16 (Figure 2C). Both Gao-Fyn and GAP43-Fyn were apparently palmitoylated to the same extent as wt Fyn, even though their expression levels were lower than wt Fyn. The reason for this is not known, although one possible explanation is that palmitoylation of the chimeric proteins is more stable than wt Fyn. These cell lines were also labeled with the iodinated myristic acid analogue IC13. Gao-Fyn was myristoylated but GAP43-Fyn was not, as predicted from their N-terminal sequences (our unpublished results).

NIH 3T3 cells expressing Gao-Fyn and GAP43-Fyn were fractionated into cytosolic (S100) and membrane (P100) fractions. Each fraction was subjected to immunoblotting. As shown in Figure 2D, Gao-Fyn and GAP43-Fyn were both present in the particulate fraction to the same extent as wt Fyn. In addition, immunofluorescence of cells expressing Gao-Fyn was performed; the chimeric protein was localized primarily to plasma membrane as well as some intracellular membranes (Figure 1G). The levels of GAP43-Fyn expression in NIH 3T3 cells were not sufficient to determine its localization by immunofluorescence. Thus, the results with the Fyn chimeras imply that the presence of palmitate can direct membrane localization of

Fyn independent of both surrounding amino acid sequence information and myristate.

The Palmitoylated N Terminus of Fyn Is Sufficient for Membrane Localization

To date, all studies examining the role of palmitoylation in the subcellular localization of Fyn have relied on loss-of-function mutations in the context of full-length Fyn protein. To determine whether the acylated N terminus of Fyn is sufficient to direct membrane localization, we constructed chimeric proteins consisting of the first 16 amino acids of wt or mutant Fyn attached to β gal, a normally cytosolic protein. The characteristics of the chimeric proteins are listed in Table 2 along with their ability to bind to membranes. The cDNAs encoding wt and mutant Fyn- β gal chimeras were transiently transfected into COS-1 cells and analyzed for palmitoylation and membrane binding ability. β gal alone was neither palmitoylated nor associated with membranes (Table 2). Attachment of the Fyn16 sequence to β gal directed palmitoylation and membrane association. However, mutation of both cysteines 3 and 6 of Fyn16- β gal abolished palmitoylation of the protein and its ability to bind to membranes (Table 2). Interestingly, the single cysteine mutant proteins, Fyn16 C3S- β gal and Fyn16 C6S- β gal were still palmitoylated, but to a much lower extent than wt Fyn16- β gal. This result implies that in COS-1 cells, cysteines 3 and 6 are both sites for palmitoylation in the Fyn16- β gal protein. Fyn16 C3S- β gal and Fyn16 C6S- β gal were bound more tightly to membranes than Fyn16 C3,6S- β gal (66% and 95%, respectively). Similar results were obtained for full-length Fyn protein in COS cells (Alland *et al.*, 1994). Thus, mutation of both cysteines in the Fyn16- β gal protein is necessary to completely abolish palmitoylation and severely hinder membrane binding.

Since β gal is a large cytosolic protein that can form a tetramer, we performed additional studies by fusing the first 25 amino acids of Fyn to a smaller carrier protein, GST. The Fyn25-GST chimera was nearly completely membrane bound (87%), and mutation of cysteines 3 and 6 slightly reduced the extent of membrane binding (78%, Table 2). We conclude from these experiments that the N-terminal Fyn sequence is sufficient to target a cytosolic protein to cellular mem-

Figure 1 (cont). Localization of wt Fyn and Fyn mutants in NIH 3T3 cells. Cells expressing wt Fyn (A and B), C3,6S Fyn (C), G2A Fyn (D), C3S Fyn (E), C6S Fyn (F), and G α -Fyn (G), or NIH 3T3 cells (H) were stained with anti-Fyn polyclonal antibody (Santa Cruz Biotechnology), followed by fluorescein-conjugated anti-rabbit IgG. In B, the Fyn antibody was preincubated with a blocking peptide corresponding to the antigenic peptide against which it was raised to control for nonspecific antibody staining. Plasma membrane staining is indicated by an arrowhead.

Figure 2. Expression and fractionation of chimeric Fyn proteins in NIH 3T3 cells. Cells expressing wt Fyn, G α o-Fyn, and GAP43-Fyn were lysed and samples were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted (A and D) or immunoprecipitated with anti-Fyn antibody followed by SDS-PAGE analysis and autoradiography (B and C). (A) Immunoblot of total cell lysates expressing chimeric Fyn proteins. (B) In vitro kinase assay of immunoprecipitations. (C) IC16 labeling of cells prior to immunoprecipitation. (D) Immunoblot of cells fractionated into soluble S100 (S) and particulate P100 (P) fractions by centrifugation at $100,000 \times g$.

branes and that palmitoylation of this targeting sequence affects binding to total cellular membranes.

The Palmitoylated N Terminus of Fyn Is Sufficient for Plasma Membrane Targeting of a Cytosolic Protein

Although Fyn16- β gal was bound to total cellular membranes, it was important to determine whether

Table 2. Characteristics of Fyn fusion proteins

Construct ^a	Myristate ^b	Palmitate ^c	% Membrane bound ^d
Fyn16- β gal	+	++	98 $\pm$ 2
Fyn16 C3, 6S- β gal	+	–	32 $\pm$ 2
Fyn16 C3S- β gal	+	–/+	66 $\pm$ 17
Fyn16 C6S- β gal	+	–/+	95 $\pm$ 2
β gal	–	–	17 $\pm$ 11
Fyn25-GST	+	++	87 $\pm$ 11
Fyn25 C3,6S-GST	+	–	78 $\pm$ 8
Fyn25 G2A-GST	–	–	5 $\pm$ 2

^aFyn fusion proteins were created by combining either the first 16 amino acids of Fyn with β gal (first four constructs) or the first 25 amino acids of Fyn with GST (last three constructs).

^bExpected modification by myristic acid based on amino acid sequence.

^cResults of labeling COS-1 cells with the palmitate analogue IC16.

^dResults of membrane fractionation based on four experiments for β gal fusion proteins and two experiments for GST fusion proteins. The first number represents the percentage in the pellet, and the second number represents the SD from a set of experiments.

this chimeric protein was properly targeted to plasma membranes. Immunofluorescence of COS-1 cells transiently transfected with wt or mutant Fyn16- β gal DNAs was used to address this issue. The micrographs presented in Figure 3 illustrate that although β gal was cytosolic, wt Fyn16- β gal was, in fact, targeted to the plasma membrane (Figure 3, A and B). COS-1 cells expressing full-length wt Fyn exhibited a pattern of plasma membrane staining similar to wt Fyn16- β gal and biochemical fractionation of wt Fyn on Optiprep density gradients has confirmed its plasma membrane localization (van't Hof and Resh, 1997). Mutation of cysteines 3 and 6 in the Fyn16- β gal chimera abolished plasma membrane staining and caused the protein to localize to the cytosol and intracellular punctate structures (Figure 3C). Mutation of cysteine 3 also abolished plasma membrane staining (Figure 3D), with staining observed in punctate intracellular membranes. Interestingly, mutating cysteine 6 had an intermediate effect. In some cells, Fyn16 C6S- β gal was localized primarily to the plasma membrane (Figure 3F), but in others, Fyn16 C6S- β gal was localized to punctate structures similar to those seen in cells expressing Fyn16 C3S or C3,6S- β gal (Figure 3E). This correlates with the fact that Fyn16 C6S- β gal is not palmitoylated to the same extent as wt Fyn16- β gal and may reflect variation in the amount of palmitoylation and subsequent plasma membrane binding of the C6S mutant in a given cell. To further verify the results with the Fyn- β gal chimeras, we also examined the subcellular localization of the Fyn25-GST chimeras. As depicted in Figure 4, Fyn25-GST yielded a distinct plasma membrane staining fluorescence. Mutation of

cysteines 3 and 6 resulted in a loss of plasma membrane staining and yielded cytosolic and punctate intracellular fluorescence. Thus, these studies strongly imply that the N-terminal region of Fyn is sufficient to target heterologous proteins to the plasma membrane and that palmitoylation of cysteines at positions 3 and 6 is necessary for this targeting ability.

Palmitoylation of Fyn and Fyn Chimeras Is Necessary and Sufficient for Association with the Detergent-insoluble Matrix

Palmitoylation of the Src family members has been shown to be important for localization to nonionic detergent-insoluble components of the plasma membrane (Rodgers *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995). The components of this detergent-insoluble fraction are varied and include caveolae, GPI-anchored proteins (Rodgers *et al.*, 1994; Schnitzer *et al.*, 1995), and cytoskeleton. We wished to determine how myristoylation and palmitoylation of Fyn independently contributed to the ability of the protein to associate with the detergent-insoluble matrix. NIH 3T3 cells expressing wt Fyn and N-terminal Fyn mutants were incubated at 4°C with a 1% Triton X-100-containing buffer to release soluble components. The remainder of the cells were then solubilized in an ionic-detergent-containing buffer. Each fraction was immunoprecipitated with anti-Fyn antibodies and analyzed by immunoblotting with anti-Fyn antibodies. The data in Figure 5A indicate that approximately 50% of wt Fyn and C6S Fyn were present in the Triton X-100-resistant matrix. When cysteines 3 and 6 or cysteine 3 alone were mutated to serine, only 25% of the protein was found in the resistant fraction. The nonacylated G2A Fyn associated even less tightly with the detergent-resistant matrix (16% resistant). Both the myristoylated and palmitoylated G α o-Fyn and the palmitoylated GAP43-Fyn were enriched in the detergent-resistant fraction, indicating that palmitoylation in the absence of myristoylation can confer detergent resistance to chimeric Fyn proteins. In addition, 73% of Fyn25-GST, but not Fyn25 C3,6S-GST or Fyn25 G2A-GST was associated with the detergent-resistant fraction (Figure 5A). These experiments establish that an N-terminal palmitoylated sequence is necessary and also sufficient to direct interaction with a Triton X-100-insoluble matrix.

The data illustrated in Figure 5A indicate that at steady state, only 50% of the total Fyn protein was associated with a Triton X-100-insoluble matrix. Since palmitoylation is required for detergent insolubility, it was possible that not all of the Fyn in a cell was palmitoylated and that only the palmitoylated form of the protein was targeted to the detergent-resistant fraction. To estimate the stoichiometry of Fyn palmitoylation, NIH 3T3 cells expressing wt Fyn were la-

Figure 3. Immunofluorescence of Fyn16-βgal chimeras in transiently transfected COS-1 cells. COS cells transfected with βgal (A), Fyn16-βgal (B), Fyn16 C3,6S-βgal (C), Fyn16 C3S-βgal (D), or Fyn16 C6S-βgal (E and F) were fixed, permeabilized, and stained with anti-βgal antibodies followed by fluorescein-conjugated anti-mouse IgG.

Figure 4. Immunofluorescence of Fyn25-GST in transiently transfected COS-1 cells. COS cells transfected with Fyn25-GST (A) and Fyn25 C3,6S-GST (B) were fixed, permeabilized, and stained with anti-GST antibodies, followed by fluorescein-conjugated anti-rabbit IgG.

beled to steady state with the iodinated palmitate analogue IC16. Cells were then separated into Triton X-100-soluble and -resistant fractions and immunoprecipitated with anti-Fyn antibodies. The data in Figure 5B show that IC16-labeled Fyn was preferentially enriched in the detergent-resistant fraction (70–80%), whereas total Fyn protein was equally distributed among the soluble and resistant fractions. These data imply that nearly half of the Fyn protein in a cell is not palmitoylated, because 50% of the protein is Triton X-100 soluble and the majority of this detergent-soluble protein is not palmitoylated.

Palmitoylation of Fyn Is Reversible

The thioester bond attaching palmitate to cysteine is a labile linkage. Other groups have shown that palmitate turns over more rapidly than the protein to which it is attached for the heterotrimeric G-proteins (Degt'yarev *et al.*, 1993; Wedegaertner and Bourne, 1994) and nitric oxide synthase (Robinson *et al.*, 1995). The biological consequence of this rapid turnover is reflected in the ability of these proteins to shift from membranes to cytosol upon treatment of cells with certain agonists that can stimulate depalmitoylation of the protein. To examine whether palmitoylation of Fyn is reversible *in vivo*, NIH 3T3 cells stably expressing wt Fyn were labeled to compare the half-life of Fyn protein versus palmitate on Fyn. Cells were labeled with [35 S]methionine to analyze total Fyn protein or with various radio-iodinated fatty acid analogues to analyze the turnover of the fatty acid modifications on Fyn. The IC13 and IC16 analogues contain 125 I at the ω carbon of the fatty acid; IC13 is an analogue of myristic acid and IC16 closely resembles palmitic acid (Peseckis *et al.*, 1993). Both of these fatty acid analogues have been used extensively by our laboratory

as sensitive and accurate probes for protein acylation. Cells were labeled to steady state with [35 S]methionine, IC16, or IC13 and were then incubated in chase medium containing a 10-fold excess of nonradioactive methionine, palmitic acid, or myristic acid, respectively. At various times after addition of chase medium, cells were lysed and Fyn was immunoprecipitated. Immunoprecipitates were electrophoresed and the Fyn band was quantitated by using PhosphorImager analysis. A representative experiment shown in Figure 6 illustrates that the half-life of Fyn protein was approximately 8 h. The half-life of the myristic acid analogue, IC13, was also approximately 8 h, an expected result since myristate is covalently attached to Fyn through an amide linkage that should be as stable as the polypeptide backbone. In contrast, the half-life of the palmitic acid analogue IC16 was 1.5–2 h. Results from several experiments indicated that the half-life of Fyn protein ranged from 8 to 12 h and the half-life of palmitate on Fyn was always 1.5–2 h. This implies that palmitoylation of Fyn is reversible *in vivo* and that palmitate attached to Fyn turns over more rapidly than Fyn protein.

DISCUSSION

In this study, we have shown that palmitoylation of Fyn is a reversible modification *in vivo* and established the functional importance of fatty acylation in dictating the subcellular localization of Fyn. This was accomplished by creating stable NIH 3T3 cell lines expressing wt and mutant forms of Fyn. In addition, chimeric Fyn proteins were constructed that allowed us to separate the role of palmitoylation from that of sequence context and N-myristoylation. Our data indicate that the presence of a cysteine residue at position 3 is important for both

Figure 5. Triton X-100 fractionation of NIH 3T3 and COS-1 cells expressing wt Fyn and various Fyn mutants and chimeras. (A) Cells were separated into Triton X-100-soluble and -resistant fractions and immunoprecipitated with anti-Fyn antibody. Samples were immunoblotted with either anti-Fyn antibodies (Transduction Laboratories) or anti-GST antibodies (Sigma) and blots were quantitated by densitometry. The graph shows the percentage of protein in the Triton X-100-resistant fraction for each construct. (B) NIH 3T3 cells expressing wt Fyn were separated into Triton X-100-soluble (S) and -resistant (R) fractions and immunoprecipitated with anti-Fyn antibodies. Left, immunoblot of Fyn immunoprecipitates. Right, cells that were labeled with IC16, fractionated, and then immunoprecipitated with anti-Fyn antibodies.

palmitoylation of Fyn and its ability to associate with plasma membrane. Cysteine 3 is conserved in the Src family members and certain members of the $G\alpha$ subunit family (Linder *et al.*, 1993). To date, all investigations, with one exception (Rodgers *et al.*, 1994), have observed that mutation of cysteine 3 decreases or prevents palmitoylation. Moreover, introduction of a cysteine at position 3 into Src promotes palmitoylation of an otherwise nonpalmitoylated protein (Alland *et al.*, 1994). Although direct chemical identification of a palmitoylated cysteine at position 3 is not yet available, the existing biochemical data strongly support the contention that cysteine 3 is the major palmitoylated residue in the Src family members.

Figure 6. Pulse-chase experiment of NIH 3T3 cells expressing wt Fyn. Cells were labeled to steady state with [^{35}S]methionine, the myristate analogue IC13, or the palmitate analogue IC16. Cells were then incubated in chase medium containing a 10-fold excess of nonradioactive methionine (Δ), myristic acid ($\blacktriangle$), or palmitic acid ($\bullet$). At various times after addition of chase medium, cells were lysed and lysates immunoprecipitated with anti-Fyn antibody. Samples were electrophoresed on 10% SDS-polyacrylamide gels. Gels were quantitated by PhosphorImager analysis. Results shown are from one representative experiment.

Palmitoylation of Fyn Specifically Influences Plasma Membrane Targeting

Several different biochemical and immunocytochemical methods were exploited to examine the subcellular localization of wt and mutant Fyn proteins. Hypotonic lysis of cells followed by ultracentrifugation revealed that nearly all of the wt Fyn (>90%) was associated with the particulate P100 fraction, in agreement with previous results (Alland *et al.*, 1994; Table 1). It is important to note that the P100 represents a crude fraction consisting of total cellular membranes. To more accurately examine the subcellular localization of Fyn, further cytological analysis was performed. Immunofluorescence revealed that wt Fyn was localized to intracellular and plasma membranes (Figure 1). Experiments in our laboratory have also shown that Fyn colocalizes with plasma membrane markers on linear density gradients (van't Hof and Resh, 1997). When the major site of palmitoylation, cysteine 3, was mutated individually or with cysteine 6, plasma membrane staining was abolished. The altered membrane association was reflected by an increased amount of C3S and C3,6S mutant proteins in the soluble cytosolic fraction (Table 1). However, nearly 60% of the nonpalmitoylated Fyn proteins still fractionated with the P100 membrane fraction. The particulate form of Fyn is likely associated with intracellular membranes, based on the punctate intracellular staining pattern. The identity of these punctate intracellular structures is currently being investigated.

To date, all reports of palmitoylation of Src family members have relied on the use of site-directed mutagenesis and subsequent loss of function (Shenoy-Scaria *et al.*, 1993, 1994; Alland *et al.*, 1994; Rodgers *et al.*, 1994). A novel feature of the present study is the use of chimeric Fyn proteins that retain palmitoylation sites in the presence or absence of myristoylation and/or Fyn-like N-terminal sequences. Two chimeric proteins, G α -Fyn and GAP43-Fyn were constructed containing heterologous sequences in place of the Fyn N-terminal region. Both chimeras were palmitoylated and exhibited membrane fractionation patterns similar to wt Fyn (Figure 2). The only feature common to the N-terminal sequence of all three proteins is the presence of palmitoylated cysteine(s). These data imply that palmitoylation of cysteine residue(s) in the N-terminal region of Fyn is required for efficient membrane association.

Two additional lines of evidence support the hypothesis that palmitoylation is not only necessary but also sufficient for plasma membrane targeting. By using a gain-of-function approach to verify the importance of the palmitate moiety, we have isolated the membrane targeting information contained within the N-terminal region of Fyn from the rest of the molecule. The first 16 amino acids of Fyn were fused to the normally cytosolic protein, β gal. The Fyn16- β gal chimera was entirely membrane bound (Table 2) and exhibited distinct plasma membrane fluorescence when expressed in COS-1 cells (Figure 3A). Mutation of cysteines 3 and 6 within Fyn16- β gal caused the protein to shift to a soluble fraction (Table 2) and resulted in a loss of plasma membrane fluorescence (Figure 3C). We have also observed that chimeras consisting of the first 25 amino acids of Fyn fused to GST (Figure 4) or the first 10 amino acids of Fyn fused to green fluorescent protein are membrane associated (our unpublished data). A similar approach was taken by Zuber *et al.* (1989), who observed that a chimeric protein containing the first 10 amino acids of GAP43 fused to chloramphenicol acetyltransferase was localized to distinct regions of the plasma membrane in neuronal cells. Thus, these data indicate that the presence of palmitoylated cysteines near the N terminus of a protein are sufficient for plasma membrane targeting.

What Is the Role of N-Myristoylation in Membrane Interaction of Fyn?

We and others have previously shown that the N-terminal region of Fyn is dually fatty acylated with both myristate and palmitate. Mutation of the N-terminal glycine to alanine (G2A) resulted in a nonmyristoylated protein that was predominantly cytosolic, based on fractionation and immunofluorescence (Table 1 and Figure 3). Myristoylation is therefore impor-

tant for membrane association of wt Fyn, a result previously reported for a variety of membrane-bound N-myristoylated proteins. However, the G2A mutation abrogates both myristoylation and palmitoylation in Fyn. The dependence of palmitoylation on N-myristoylation has been noted for Fyn, Lck, Hck, and several G α subunits (Schaller *et al.*, 1992; Alland *et al.*, 1994; Robbins *et al.*, 1995; Schnitzer *et al.*, 1995). Use of the GAP43-Fyn chimera allowed us to separate the role of palmitoylation from N-myristoylation. The GAP43 sequence is palmitoylated but not myristoylated, and GAP43-Fyn associated with membranes in a manner similar to wt Fyn (Figure 2). Thus, myristoylation per se is not an absolute requirement for palmitoylation or membrane association of Fyn. It is likely that the role of myristate is to enhance accessibility of Fyn to a membrane-bound palmitoyl acyltransferase(s). Indeed, we and others have observed palmitoylation of nonmyristoylated Fyn (G2A) and G α proteins that are anchored to the membrane by alternate membrane targeting domains (Degtyarev *et al.*, 1994; our unpublished data).

The present studies of Fyn and Fyn chimeras, coupled with the work of our laboratory and others on fatty acylated and lipid-modified proteins, provide further support for the two-signal model of membrane association (Hancock *et al.*, 1990; Resh, 1994). The presence of myristate plus palmitate (wt Fyn, G α -Fyn) or two palmitates (GAP43-Fyn) can direct a protein to membranes. Proteins such as Src and human immunodeficiency virus-1 Gag use myristate plus a polybasic domain to achieve plasma membrane binding (Buser *et al.*, 1994; Sigal *et al.*, 1994; Zhou *et al.*, 1994). Interestingly, Kwong and Lublin (1995) recently generated a membrane-bound Lck construct containing myristate plus a polybasic sequence. In the Ras family, a C-terminal farnesyl moiety (equivalent in hydrophobicity to myristate) functions in conjunction with either a polybasic domain (K-Ras4B) or a palmitoylated cysteine(s) to direct Ras to the plasma membrane (Hancock *et al.*, 1990; Cadwallader *et al.*, 1994). Thus, two modular elements are necessary for membrane association: myristate/farnesyl plus palmitate/polybasic domain.

Palmitoylation of Fyn Is Necessary and Sufficient for Association with a Detergent-insoluble Matrix

Previous experiments with Fyn and Hck have shown that palmitoylation of cysteines in the N terminus of the protein is necessary for localization to detergent-resistant portions of the plasma membrane, termed the GEM fraction (Rodgers *et al.*, 1994), which is enriched in GPI-anchored proteins, glycosphingolipids, and caveolae (Shenoy-Scaria *et al.*, 1993, 1994; Schnitzer *et al.*, 1995). Herein, we have further defined the requirements for Fyn association with a Triton

X-100-resistant fraction. Approximately 50% of wt Fyn associated with the Triton X-100-resistant fraction (Figure 5A), but only 25% of nonpalmitoylated Fyn associated with this fraction. Neither cytochalasin D, which disrupts the actin cytoskeleton, nor nocodazole, which interferes with microtubule polymerization, had any effect on the ability of Fyn to partition with the detergent-insoluble matrix (Wolven and Resh, unpublished data). The exact components of this detergent-resistant fraction that interact with Fyn remain to be identified. One interesting aspect of the data presented herein is that GAP43-Fyn, which is palmitoylated but not myristoylated, interacts quite strongly with the detergent-resistant matrix (Figure 5A). This may indicate that palmitoylation itself is sufficient for this interaction without a requirement for myristoylation or specific N-terminal sequence information. Additional evidence that palmitoylation of Fyn may be specifically mediating an interaction with components of a Triton X-100-insoluble fraction comes from the finding that the palmitoylated form of Fyn is preferentially enriched in this fraction (Figure 5B). We have also observed that a chimeric protein consisting of the first 25 amino acids of Fyn fused to GST is Triton X-100 insoluble (Figure 5A). The evidence presented herein provides for a model in which the acylated N terminus of Fyn is sufficient for plasma membrane targeting and an additional interaction with specific subdomains of the plasma membrane that are Triton X-100 insoluble.

Plasma Membrane Targeting via "Bilayer Trapping"

The data presented in this study are consistent with the following model for membrane targeting of dually fatty acylated proteins. After synthesis and myristoylation on cytoplasmic polysomes (van't Hof and Resh, 1997), myristoylated Fyn can transiently interact with multiple intracellular membranes. However, those Fyn molecules that reach the plasma membrane will be palmitoylated by a plasma membrane-bound palmitoyltransferase (Dunphy *et al.*, 1996) and become "trapped" due to the strong hydrophobicity and slow "off" rate provided by the presence of both myristate and palmitate. The experimental data showing plasma membrane localization of wt and chimeric Fyn proteins (this study) and dually acylated myr-Gly-Cys containing peptides (Schroeder *et al.*, 1996) strongly support the "bilayer trapping" model originally proposed by Shahinian and Silviu (1995).

The forces that retain Fyn at the plasma membrane could be a combination of nonspecific hydrophobic interactions of the fatty acids with the lipid bilayer and potential specific interactions between Fyn and other membrane proteins. Although myristoylation alone does not provide sufficient energy for stable membrane binding (Peitzsch and McLaughlin, 1993), studies with pure lipid vesicles

have established that the combination of myristate plus palmitate is sufficient to retain Fyn at a lipid bilayer, even in the absence of any other proteins (Shahinian and Silviu, 1995). In the eukaryotic cell, it is known that Fyn forms additional protein-protein interactions at the plasma membrane. For example, Fyn has been shown to associate with a number of transmembrane-spanning cell surface receptors, including Ig α (Campbell and Sefton, 1992; Clark *et al.*, 1992, 1994; Pleiman *et al.*, 1994), Fc receptors (Sugie *et al.*, 1991; Sarmay *et al.*, 1994), CD36 (Huang *et al.*, 1991), the platelet-derived growth factor (PDGF) receptor (Kypka *et al.*, 1990; Twamley *et al.*, 1992; Mori *et al.*, 1993; Twamley-Stein *et al.*, 1993), and several T-cell receptor subunits (Samelson *et al.*, 1990; Gauen *et al.*, 1992). Association of Fyn with the T-cell receptor requires the presence of glycine 2, cysteine 3, and two additional lysines in the SH4 motif (Gauen *et al.*, 1996). These same residues were also shown to be necessary for efficient fatty acylation and plasma membrane targeting. We have determined that myristoylated glycine 2 and palmitoylated cysteine 3 within Fyn are required for interaction between Fyn and the activated PDGF receptor (Wolven and Resh, unpublished data). It is therefore likely that the function of the dually acylated SH4 motif is to increase the local concentration of Fyn at the plasma membrane, thereby enhancing the efficiency of interaction with other plasma membrane signaling proteins.

Palmitoylation of Fyn Is a Reversible Modification

We have shown herein that the removal of palmitate on Fyn occurs *in vivo* before the protein is degraded. Pulse-chase studies with NIH 3T3 cells stably expressing wt Fyn revealed that the half-life of the Fyn protein was 8–12 h, whereas the half-life of the palmitate attached to Fyn was 1.5–2 h (Figure 6). A comparison of the half-life of myristate versus palmitate on Fyn revealed that myristate remains attached to Fyn during the lifetime of the protein, whereas palmitate is removed on this time scale. This is not surprising considering that myristate is covalently attached via an amide linkage, and palmitate is linked via a hydrolyzable thioester bond. These data raise the interesting possibility that depalmitoylation of Src family members may be regulated. Other groups have reported that the α_s subunit of the heterotrimeric G proteins is reversibly palmitoylated and that this modification is regulated by stimulation with an agonist of the β -adrenergic receptor, isoproterenol (Degtyarev *et al.*, 1993; Wedegaertner and Bourne, 1994). Stimulation by isoproterenol leads to a shift from a membrane bound to a cytosolic form of the protein, presumably mediated by depalmitoylation of α_s subunits. Evidence for this

type of regulatory mechanism also exists for endothelial nitric oxide synthase (Robinson *et al.*, 1995). We have attempted to determine whether regulated depalmitoylation can occur with Fyn by stimulating cells with serum growth factors or PDGF (Wolven and Resh, unpublished data). However, under these conditions we have seen no difference in the kinetics of loss of palmitate from Fyn. This does not rule out the possibility that regulated depalmitoylation of Fyn does not occur. Experiments are currently underway to determine whether other stimuli in other systems can stimulate depalmitoylation and relocalization of Fyn.

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