Dual Fatty Acylation of p59Fyn Is Required for Association with the T Cell Receptor z Chain through Phosphotyrosine-Src Homology Domain-2 Interactions

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Abstract. The first 10 residues within the Src homology domain (SH)-4 domain of the Src family kinase Fyn are required for binding to the immune receptor tyrosine-based activation motif (ITAM) of T cell receptor (TCR) subunits. Recently, mutation of glycine 2, cysteine 3, and lysines 7 and 9 was shown to block binding of Fyn to TCR z chain ITAMs, prompting the designation of these residues as an ITAM recognition motif (Gauen, L. K. T., M. E. Linder, and A. S. Shaw. 1996. J. Cell Biol. 133:1007–1015). Here we show that these residues do not mediate direct interactions with TCR ITAMs, but rather are required for efficient myristoylation and palmitoylation of Fyn. Specifically, coexpression of a K7,9A-Fyn mutant with N-myristoyltransferase restored myristoylation, membrane binding, and association with the cytoplasmic tail of TCR z fused to CD8. Conversely, treatment of cells with 2-hydroxymyristate, a myristoylation inhibitor, blocked association of wild-type Fyn with z. The Fyn NH2 terminus was necessary but not sufficient for interaction with z and both Fyn kinase and SH2 domains were required, directing phosphorylation of z ITAM tyrosines and binding to z ITAM phosphotyrosines. Fyn/z interaction was sensitive to octylglucoside and filipin, agents that disrupt membrane rafts. Moreover, a plasma membrane bound, farnesylated Fyn construct, G2A,C3S-FynKRas, was not enriched in the detergent insoluble fraction and did not associate with z. We conclude that the Fyn SH4 domain provides the signals for fatty acylation and specific plasma membrane localization, stabilizing the interactions between the Fyn SH2 domain and phosphotyrosines in TCR z chain ITAMs.

Key words: Src homology domains • acylation • cell membrane • protein-tyrosine kinase • receptor/antigen

Proteins belonging to the Src family of nonreceptor tyrosine kinases critically rely on binding to the cytoplasmic leaflet of the plasma membrane lipid bilayer to fulfill their biological functions (32, 40). Membrane targeting is specifically guided by the Src homology domain (SH)-4 motif, a short sequence in the NH2-terminal region of Src family kinases (30, 31). SH4 motifs contain two independent signals (myristate and either palmitate or a polybasic amino acid sequence) that control stable membrane attachment when acting together, but not when operating individually (31, 32). For example, specific and rapid plasma membrane targeting of the Src family kinase Fyn is encoded by dual fatty acylation with myristate at glycine 2 and palmitate at cysteine 3 (1, 35, 41, 43).

Protein myristoylation involves the cotranslational attachment of the 14 carbon fatty acid myristate to the NH2-terminal glycine (glycine 2) of target proteins (8, 17, 42). A chemically stable amide bond is formed between myristate and glycine and is maintained for the lifetime of the protein. The enzymatic reaction is catalyzed by N-myristoyltransferase (NMT), a cytosolic protein that is ubiquitously expressed in eukaryotic cells. The minimum consensus sequence for N-myristoylation is GXXXS/T; glycine 2 is a critical component and a serine or threonine at position 6 is preferred (17). Additional amino acids have been shown to be important for recognition by NMT in the context of specific protein sequences. N-myristoylation of Fyn is a prerequisite for posttranslational and dynamic palmit-
toylation to occur. Mutations that ablate myristoylation result in loss of palmitoylation (1, 35, 41, 43). Efficient myristoylation of Fyn, therefore, is essential for correct processing and intracellular targeting of Fyn (41, 43).

One of the functional consequences of dual fatty acylation and plasma membrane targeting is to allow Src family kinases to interact with transmembrane receptor complexes. For example, Fyn coimmunoprecipitates with the activated T cell receptor (TCR) and has been reported to interact with multiple TCR subunits (10, 33). Fyn tyrosine kinase activity is stimulated when the TCR is cross-linked, but the molecular nature of the Tyn/TCR interaction is not entirely clear, as the complex is only detected by the use of mild detergents to lyse cells (10, 33, 40). The presence of immune receptor tyrosine-based activation motifs (ITAM) on the chain of the TCR was shown to be essential for interaction with Fyn, although no specific amino acid within the ITAM seemed to be responsible for this interaction (12). Recently, mutation of glycine 2, cysteine 3, and lysines 7 and 9 within the SH 4 domain of Fyn was reported to block binding to the chain of the TCR, prompting the designation of these four residues as an ITAM recognition motif (11). However, glycine 2 is required for myristoylation and cysteine 3 is the main site of palmitoylation in Fyn (1, 35, 43). In addition, lysine 7 has been implicated in directing efficient myristoylation of Src (6, 19). Thus, it remained possible that alteration in fatty acylation levels was responsible for the inability of the mutant Fyn to interact with chain.

We therefore reevaluated the role of the SH 4 motif of Fyn, focusing on the significance of lysines 7 and 9 in fatty acylation and membrane targeting of Fyn, and its interactions with the TCR chain. Here we show that dual fatty acylation of a Fyn mutant was drastically impaired. A gain of function experiment revealed that overexpression of NMT restored fatty acylation levels of A-Fyn mutant was drastically impaired. Thus, it remained possible that alteration in fatty acylation levels was responsible for the inability of the mutant Fyn to interact with chain.

Materials and Methods

Materials

Cell culture reagents were purchased from Gibco Laboratories. cDNA’s for human wild-type Fyn, G-Fyn, and C-S-Fyn (11) and NMT (28) and rabbit polyclonal antisera raised against Fyn SH 4 protein (3) were from lab stocks. E. coli O157-T76, and CD8-4F (16, 22) were kindly provided by Dr. A. Rahr Weiss (Department of Immunology, University of California, San Francisco, San Francisco, CA). pSV LSH 2-Fyn, containing mutant Fyn lacking amino acids 144-248, was kindly provided by Drs. Nicolas Deunant and K. Kurt Balmer-Hofer (Friedrich Miescher Institute, Basel, Switzerland). pEGFP N1 and rabbit polyclonal antibody against green fluorescent protein (GFP) were obtained from Clontech. An nti-CD8 monoclonal antibody solution and high purity dityrosine were from Calbiochem. Protein A-agarose and protein A (agarose beads and antiphosphotyrosine (PY 99) monoclonal antibody solution were purchased from Santa Cruz Biotechnology. DL-hydroxyymristic acid (2-hydroxy-tetradecanoic acid), defatted BSA, and filipin were from Sigma Chemical Co. and n-octylglucoside was from Boehringer Mannheim. Transfomable and cell line, to which the Fyn SH 2 domain was subcloned into pCMV5, as described above. K-Fyn constructs, sense oligonucleotides were synthesized to encode the upstream region of pGEM 3Z, an NcoI site, and either the first 11-14 amino acids of human Fyn, containing lysines to alanine substitutions at positions 7 and/or 9, or the first 10 amino acids of human Lck, followed by amino acids 11-16 of human Fyn. An antisense oligonucleotide was used corresponding to a region of the SH 3 domain of Fyn (1). Sense and antisense primers were used with pGEM 3Z-Fyn as a template to generate mutant cDNA’s, which were digested with NcoI and BclI to produce 7-8 bp fragments that were used to replace the corresponding fragments of Fyn in pSP65. A G-FynK R construction was generated by PCR using a sense oligonucleotide encoding nucleotides 1110-1130 of human Fyn containing a unique BglII site, and an antisense oligonucleotide corresponding to the last 18 nucleotides of Fyn, followed by the last 5 nucleotides of KRas 4B, a stop codon, and a SalI site. The primers were used with pGEM 3Z-Fyn as a template. The PCR product was digested with BglII and SalI to produce a 500-bp fragment that was used to replace a corresponding carboxy-terminal fragment of G-Fyn in pSP65. Two Fyn constructs in pSP65 were subsequently digested with EcoRI and BglII, and ligated into pCMV5. FynN constructs was subsequently digested by generation of pCMV S-Ga-Fyn K R as with BgII and SalI, followed by ligation into BglII cut pCMV 5-Fyn. G-A, C-S-Fyn R as was generated by PCR, with pGEM 3Z-Fyn as a template, using a sense oligonucleotide encoding an NcoI site following the first eight amino acids of human Fyn, containing a glycineto-alanine substitution at position 2 and a cysteine-to-serine substitution at position 3, and an antisense oligonucleotide corresponding to the SH 3 domain of Fyn (1). The PCR product was digested with NcoI and BstXI to produce a 7-8 bp fragment that was used to replace the corresponding fragment of pSP65-FynK R as. G-A, C-S-Fyn R as was subcloned into pCMV 5 as described above. DH2-Fyn was subcloned into pSP65 by digestion of pSv DH2-Fyn with BstXI and BstE I and ligation into BstXI and BstE I-digested pSP65, and subcloned into pCMV 5, as described above. K-Fyn constructs were used with pEGFP N1 as a template to generate a 1.6 kb DNA fragment that was digested with EcoRI and XbaI, followed by ligation into pCMV5.

Plasmid Construction

Construction of plasmids containing wild-type Fyn, G-Fyn, C-S-Fyn, and G-A-Fyn was described before (1, 41). For generation of K-Fyn, K-A-Fyn, K-A-Fyn, and Lck(10)Fyn constructs, sense oligonucleotides were synthesized to encode the upstream region of pGEM 3Z, an NcoI site, and either the first 11-14 amino acids of human Fyn, containing lysines to alanine substitutions at positions 7 and/or 9, or the first 10 amino acids of human Lck, followed by amino acids 11-16 of human Fyn. An antisense oligonucleotide was used corresponding to a region of the SH 3 domain of Fyn (1). Sense and antisense primers were used with pGEM 3Z-Fyn as a template to generate mutant cDNA’s, which were digested with NcoI and BstXI to produce 7-8 bp fragments that were used to replace the corresponding fragments of Fyn in pSP65. A G-A-FynKR construction was generated by PCR using a sense oligonucleotide encoding nucleotides 1110-1130 of human Fyn containing a unique BglII site, and an antisense oligonucleotide corresponding to the last 18 nucleotides of Fyn, followed by the last 5 nucleotides of KRas 4B, a stop codon, and a SalI site. The primers were used with pGEM 3Z-Fyn as a template. The PCR product was digested with BglII and SalI to produce a 500-bp fragment that was used to replace a corresponding carboxy-terminal fragment of G-A-Fyn in pSP65. A II Fyn constructs in pSP65 were subsequently digested with EcoRI and SalI, and ligated into pCMV5. FynN constructs was subsequently digested by generation of pCMV S-Ga-Fyn K R as with BgII and SalI, followed by ligation into BglII cut pCMV 5-Fyn. G-A, C-S-Fyn R as was generated by PCR, with pGEM 3Z-Fyn as a template, using a sense oligonucleotide encoding an NcoI site following the first eight amino acids of human Fyn, containing a glycineto-alanine substitution at position 2 and a cysteine-to-serine substitution at position 3, and an antisense oligonucleotide corresponding to the SH 3 domain of Fyn (1). The PCR product was digested with NcoI and BstXI to produce a 7-8 bp fragment that was used to replace the corresponding fragment of pSP65-FynK R as. G-A, C-S-Fyn R as was subcloned into pCMV 5 as described above. DH2-Fyn was subcloned into pSP65 by digestion of pSv DH2-Fyn with BstXI and BstE I and ligation into BstXI and BstE I-digested pSP65, and subcloned into pCMV 5, as described above. K-Fyn constructs were used with pEGFP N1 as a template to generate a 1.6 kb DNA fragment that was digested with EcoRI and XbaI, followed by ligation into pCMV5.

Cell Culture and Transfection

COS-1 cells (A merican Type Culture Collection) were cultured and transfected as previously described (41). For cotransfection experiments with Fyn and NMT, 2 µg Fyn cDNA was used with 4 or 10 µg NMT cDNA (ratios 1:2 and 1:5). For CD8-γ coimmunoprecipitation experiments, 5 µg...
Metabolic Radiolabeling, Cell Fractionation and Pretreatment with 2-Hydroxymyristate and Filipin

Transfected COS-1 cells were starved for 1 h at 37°C in DMEM minus methionine/cysteine, supplemented with 2% dialyzed FBS, and labeled for 4 h at 37°C with 25 μCi/ml Tran35S-label. For radiolabeling with fatty acid analogues, cells were starved for 1 h at 37°C in DMEM containing 2% dialyzed FBS, followed by radiolabeling for 4 h at 37°C with either 25 μCi/ml 129I-I-IC13 or 129I-I-IC16 (1, 41). Fractionation into cytosolic (S300) and total membrane (P100) fractions and extraction with the nonionic detergent Triton X-100 was performed as described previously (41). 2-Hydroxymyristate was stored as a 100-nM stock solution in DMSO and used for experiments at 1 mM in DMEM containing 1% defatted BSA. Before addition to cells, 1 mM 2-hydroxymyristate solution was sonicated briefly and filtered to remove any undissolved myristate analogue. After preincubation for 2 h at 37°C, DMEM containing 5% FBS was added, followed by overnight incubation at 500 μM 2-hydroxymyristate. Where indicated, cells were pretreated for 1 h at 37°C with 10 μg/ml filipin in DMEM containing 1% defatted BSA.

Immunoprecipitation, Gel Electrophoresis, and Quantitation of Fatty Acylation

For immunoprecipitation, each clarified lysate in RIPA buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate) was mixed with 3 μl anti–Fyn or 3 μl anti–GFP and 10 μl protein A–agarose solution, and incubated for 2–12 h at 4°C. Immunoprecipitates were washed twice with RIPA buffer, once with STE (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA), resuspended in SDS sample buffer containing 0.1 M DTT, and applied to SDS-PAGE. Quantitation of radiolabeled proteins was performed using the ImageQuant software provided with the PhosphorImager system (Molecular Dynamics, Inc.). In each labeling experiment, constructs were labeled in duplicate with 129I-I-IC13, 129I-I-IC16, or Tran35S-label, and the mean of the duplicate values was used as the value for each individual experiment. Numbers were corrected for the background in each individual lane of the gel. Efficiency of myristoylation was calculated as 129I-I-IC13 incorporation per unit of Tran35S-label, which is accurate since all Fyn constructs used in this report (see Table I) contain identical amounts of methionines and cysteines. Numbers for myristoylation of mutant constructs were expressed relative to wild-type Fyn within each experiment. Each construct was analyzed in two to four experiments.

CD8-ζ Immunoprecipitation Experiments

10% wt/vol stock solutions of high purity digitonin (Calbiochem Corp.) were prepared according to the manufacturer’s instructions. A fraction prolonged storage at 4°C, stocks produce precipitates that were removed by heating and filtration. For experiments, digitonin lysis buffer (1% digitonin, 25 mM Tris, pH 8.0, 150 mM NaCl, 300 mM KCl, 25 mM NaF, 0.1 mM Na3VO4) was prepared freshly using a clear 10% digitonin stock. Transiently transfected COS-1 cells were lysed for 15 min at 4°C in digitonin buffer supplemented with protease inhibitors (1.5 μg/ml of each leupeptin, aprotinin, and pepstatin A [Boehringer Mannheim], 20 μg/ml each of AEBSF, TLCK, TPECK, and benzamidine [Calbiochem Corp.]). Where indicated, the digitonin lysis buffer was supplemented with 60 mM n-octylglucoside. Lysates were centrifuged for 15 min at top speed in an Ependorf microfuge at 4°C and incubated for 4 h at 4°C with 2 μl anti–CD8 antibody solution and 10 μl protein A/G-agarose, or with 10 μl protein A/G-agarose alone. The anti–CD8 immunoprecipitates were centrifuged for 2 min at top speed in an Ependorf centrifuge, washed twice with digitonin buffer, and once with STE buffer (10 mM Tris, pH 7.4, 150 mM NaCl). Western blotting analysis for Fyn was performed as described (43), using a rabbit anti–Fyn polyclonal in the primary antibody step. To evaluate the levels of CD8-ζ expression, Fyn blots were stripped and reprobed with monoclonal anti–CD8 antibody. A controls for total Fyn expression, supernatants of the anti–CD8 immunoprecipitation step were supplemented with 5× RIPA buffer, 3 μl rabbit anti–Fyn antibody, 10 μl protein A-agarose and incubated for >4 h at 4°C. Immunoprecipitates were washed three times in RIPA buffer and subjected to Western blotting using monoclonal antibody against Fyn (Transduction Laboratories) (43).

Results

Replacement of Lysines 7 and 9 in Fyn Results in Reduced Fatty Acylation and Membrane Anchoring

The first set of experiments was designed to quantitatively assess the contributions of lysine residues within the Fyn SH4 motif towards fatty acylation. COS-1 cells were transfected with cDNAs encoding either wild-type Fyn, G3A-Fyn, or the K7,9A-Fyn construct, in which lysines at positions 7 and 9 are replaced by alanine residues (Table I). Radiolabeling with Tran35S-label, followed by immunoprecipitation of Fyn, SDS-PAGE and autoradiography, showed that all constructs were expressed at similar levels (Fig. 1). The K7,9A-Fyn construct displayed moderately elevated levels of expression and migrated as a doublet (Fig. 1). Wild-type Fyn was labeled with both 129I-I-IC13 (a myristate analogue) and 129I-I-IC16 (a palmitate analogue), while G3A-Fyn was not (Fig. 1, and Table II), as described previously (1, 41, 43). A though the K7,9A-Fyn construct contains both the glycine at position 2 and the cysteine at position 3 required for efficient myristoylation and palmitoylation of Fyn (1, 41, 43), levels of 129I-I-IC13 and 129I-I-IC16 incorporation were drastically reduced (Fig. 1). In subsequent experiments, K7A-Fyn and K9A-Fyn mutants, containing alanine replacements of either lysine 7 or 9, were included to analyze the relevance of the individual lysines for fatty acylation. Radiolabeling with Tran35S-label and 129I-I-IC13, followed by calculation of expression levels and relative myristoylation (Table II), showed that mutation of lysine 7 or 9 individually reduced myristoylation of Fyn by 51 and 40%, respectively (Table II). Myristoylation of the double mutant, K7,9A-Fyn, was inhibited by 83% (Table II), implying that the effect of each lysine mutation on myristoylation is additive. In agreement with the dependence of palmitoylation on myristoylation, the level of 129I-I-IC16-palmitate labeling of K7,9A-Fyn was also found to be reduced to 22 ± 3% (n = 4) of wild-type levels.

Lck is the only Src family member that lacks lysines at positions 7 and 9 (36, 37). Quantitative analysis of fatty acylation of full length Lck was hampered by inconsistent immunoprecipitation results using different batches of
Figure 1. Fatty acylation of Fyn, G_{A,Fyn}, and K_{7,9-A-Fyn} in COS-1 cells. COS-1 cells were transfected with cDNA encoding wild-type Fyn, G_{A,Fyn}, and K_{7,9-A-Fyn}, and radiolabeled with either Tran^{35}S-label, Tran^{125}I-IC13, or Tran^{125}I-IC16. Cells lysates were immunoprecipitated with anti-Fyn antibody and analyzed by SDS-PAGE and autoradiography. Gels were exposed to film for 8–24 h, and strips corresponding to the region of the gel containing p59^{Fyn} are shown. No other radiolabeled proteins were apparent on the gel.

Figure 2. Fatty acylation and subcellular distribution of Fyn, G_{A,Fyn}, and K_{7,9-A-Fyn} in COS-1 cells. (A) Transfected cells were radiolabeled with either Tran^{35}S-label, Tran^{125}I-IC13, or Tran^{125}I-IC16. Cells were homogenized and fractionated into S100 (S) and P100 (P) fractions, followed by immunoprecipitation, SDS-PAGE, and autoradiography. Gels were exposed to film for 8–24 h. (B) Quantitation of the subcellular distribution of 35S-labeled wild-type and mutant Fyn by PhosphorImaging. Gels were exposed to PhosphorImager screens for 8–24 h. Values represent the mean of four independent experiments.

Table II. Relative Myristoylation Levels of Wild-Type and Mutant Fyn Constructs in COS-1 Cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>125I-IC13 incorporation per 35S-labeled unit of Fyn protein</th>
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<tr>
<td></td>
<td>Tran^{35}S-label</td>
<td>Tran^{125}I-IC13</td>
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<tr>
<td>Fyn</td>
<td>100%</td>
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<tr>
<td>G_{A,Fyn}</td>
<td>87 ± 5</td>
<td>1 ± 1</td>
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<tr>
<td>K_{A,Fyn}</td>
<td>145 ± 8</td>
<td>73 ± 6</td>
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<tr>
<td>K_{A,Fyn}</td>
<td>195 ± 6</td>
<td>116 ± 8</td>
</tr>
<tr>
<td>K_{7,9-A,Fyn}</td>
<td>160 ± 12</td>
<td>28 ± 6</td>
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<tr>
<td>Lck(10)Fyn</td>
<td>125 ± 3</td>
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COS-1 cells were transfected with Fyn cDNA as indicated and radiolabeled as described in Fig. 1. Gels were exposed to PhosphorImager screens for 24 or 48 h, and incorporation of Tran^{35}S-label or Tran^{125}I-IC13 into wild-type and mutant Fyn was measured using the ImageQuant software provided with the PhosphorImager system. Values for 125I-IC13 incorporation were derived using a standard curve established with known amounts of labeled Fyn. Values for 125I-labeled Fyn were calculated relative to wild-type Fyn, and values for Tran^{35}S-labeled Fyn were calculated relative to Tran^{125}I-IC13-labeled Fyn. Background numbers comprised 5–10% of total values for both labeling procedures. Incorporation of radiolabel into the Fyn constructs is expressed relative to wild type. n, number of experiments performed for each construct.

Table II. Relative Myristoylation Levels of Wild-Type and Mutant Fyn Constructs in COS-1 Cells

Since fatty acylation of Fyn is essential for membrane binding (1, 41, 43), the reduced levels of fatty acid modification of K_{7,9-A-Fyn} would be expected to alter the subcellular distribution of this construct. Transfected cells expressing the various Fyn constructs were radiolabeled with Tran^{35}S-label, Tran^{125}I-IC13 or Tran^{125}I-IC16, followed by homogenization and fractionation into high-speed supernatant (S100) and membrane (P100) fractions, and the distribution of Fyn was analyzed. Fig. 2 shows that more than 95% of wild-type Fyn was recovered from the membrane fraction as dually acylated protein, whereas approximately 75% of G_{A,Fyn} was recovered from the soluble fraction, in agreement with previous results (1, 41, 43). In addition, slightly more than 50% of K_{7,9-A-Fyn} was found in the soluble fraction (Fig. 2B), specifically consisting of the protein band with the slower migration, that is neither myristoylated nor palmitoylated (A). The reason for this difference in migration is not clear, and it cannot be explained simply by the absence of fatty acylation since the G_{A,Fyn} mutant does not show this effect. The remainder of K_{7,9-A-Fyn} was recovered from the membrane fraction as dually acylated protein (Fig. 2A). The calculated ratio of Tran^{125}I-IC13 label to Tran^{35}S-labeled in the P100 fraction was 25% lower for K_{7,9-A-Fyn} compared with wild-type Fyn (data not shown), implying that some of the protein in the membrane fraction is not acylated. This is confirmed by the results with G_{A,Fyn}, for which approximately 25% of the protein is found in the membrane pellet, even though none of the protein is fatty acylated (Fig. 2 and Table II).

Overexpression of Enzymatically Active NMT Rescues the Impaired Fatty Acylation and Membrane Binding of K_{7,9-A-Fyn}

Cotransfection experiments were performed to investigate whether the reduced myristoylation of the K_{7,9-A-Fyn} mutant was related to levels of NMT activity inside cells. COS-1 cells were transfected with Fyn or K_{7,9-A-Fyn} cDNA alone (Fig. 3, lanes 1 and 2), or cotransfected with cDNA encoding either wild-type NMT (lanes 3 and 4) or...
H218N-NMT (lanes 5 and 6). H218N-NMT contains a point mutation in the active site that abolishes transfer of myristoyl-CoA to protein (28), and serves as a negative control for the requirement of NMT activity. The ratio of NMT cDNA to Fyn cDNA used for transfection was 2:1. Fig. 3 shows that Fyn (lanes 1, 3, and 5) and K7,9A-Fyn protein (lanes 2, 4, and 6) were expressed at similar levels, with K7,9A-Fyn exhibiting an extra band with a slower migration and reduced myristoylation relative to wild-type Fyn (compare lanes 1 and 2). Cotransfection of K7,9A-Fyn with wild-type NMT increased 125I-IC13 labeling two- to threefold (compare Fig. 3, lanes 1 and 2 with lanes 3 and 4, and see Fig. 4 B), whereas cotransfection with enzymatically inactive H218N-NMT had no effect (compare Fig. 3, lanes 1 and 2 with lanes 5 and 6). Cotransfection of K7,9A-Fyn with wild-type NMT also increased 125I-IC16 incorporation of K7,9A-Fyn twofold (data not shown). In addition, the relative intensity of the lower band in the K7,9A-Fyn doublet increased (Fig. 3, lane 4), consistent with an increase in fatty acylation levels.

The data in Figs. 1–3 imply that levels of endogenous NMT in COS-1 cells are limiting for myristoylation of K7,9A-Fyn. To verify this supposition, the effect of varying the ratio of NMT cDNA to Fyn cDNA was tested. Myristoylation levels of K7,9A-Fyn increased twofold, using twice the amount of NMT cDNA and three- to fivefold using five times more NMT cDNA (Fig. 4 A). No significant effect on myristoylation of wild-type Fyn was observed (Fig. 4 A), implying that the amount of endogenous NMT was sufficient to achieve maximal myristoylation levels of this protein. A nalysis of NMT expression by Western blotting confirmed that increasing levels of NMT protein were expressed after cotransfection with NMT cDNA (data not shown). Radiolabeling with Tran35S-label, followed by subcellular fractionation, showed that the distribution of wild-type Fyn between the soluble and the membrane fraction was not affected during coexpression of NMT.
In contrast, K_{7,9A} -Fyn expressed in the presence of NMT displayed a significant shift from the soluble to the membrane fraction (Fig. 4C). Taken together, these experiments demonstrate that the fatty acylation defect of the K_{7,9A} -Fyn mutant can be restored by expressing excess amounts of exogenous NMT.

**Coimmunoprecipitation of Fyn with the ζ Chain of the TCR Depends on the Fatty Acylation Status of Fyn**

The ability to manipulate fatty acylation of Fyn provided us with a unique opportunity to quantitate the individual contributions of the fatty acids and the surrounding amino acids towards interactions of Fyn with other membrane bound proteins. We chose to evaluate the interactions of Fyn with the ζ chain of the TCR complex as a model system. COS-1 cells were cotransfected with cDNAs for Fyn and CD8-ζ, a chimeric construct containing the extracellular and transmembrane regions of CD8, fused to the cytoplasmic tail of the TCR ζ chain (16). Cells were lysed in a 1% digitonin lysis buffer (10, 33) and subjected to immunoprecipitation with anti-CD8 monoclonal antibody. Incubation with protein A/G+ agarose beads alone was used as a negative control. When anti-CD8 immunoprecipitates were analyzed by Western blotting using polyclonal anti-Fyn antibody, a signal for Fyn was obtained using lysates from cells coexpressing Fyn and CD8-ζ (Fig. 5A, lane 3). Relative to the amount of wild-type Fyn, only marginal amounts (<5%) of G_{2A}-Fyn and C_{3,6S}-Fyn were detected after immunoprecipitation with anti-CD8 antiseraum (Fig. 5, A, lanes 5 and 7, and B). Since G_{2A}-Fyn is neither myristoylated nor palmitoylated and C_{3,6S}-Fyn is myristoylated but not palmitoylated (1, 43), these data indicate that fatty acylation of Fyn with both myristate and palmitate

![Figure 5. Coimmunoprecipitation of wild-type or mutant Fyn constructs with CD8-ζ. (A) COS-1 cells were cotransfected with cDNAs encoding wild-type or mutant Fyn (see Table I), CD8-ζ, and NMT where indicated. Cells were lysed in digitonin buffer and subjected to immunoprecipitation with anti-CD8 monoclonal antibody (+) or with protein A/G+ agarose beads alone (−). Immunoprecipitates were analyzed by SDS-PAGE and Western blotting using polyclonal anti-Fyn antibody (top). Subsequently, the blot was stripped and reprobed using anti-CD8 monoclonal antibody; the distribution of the 41-kD form of CD8-ζ is shown (middle). After immunoprecipitation with anti-CD8 antibody, lysates were subjected to a second immunoprecipitation step, using polyclonal anti-Fyn antibody, followed by SDS-PAGE and Western blotting using monoclonal anti-Fyn antibody (bottom). (B) Levels of Fyn protein coimmunoprecipitating with CD8-ζ were quantified by scanning of films. Films were exposed for different times to the blot and measurements were made in the linear range of the film. The measured values for coimmunoprecipitation with CD8-ζ were corrected for total expression levels and values are expressed relative to the amount of wild-type Fyn that coimmunoprecipitates with CD8-ζ. Given values represent the mean of two experiments.](image-url)
Inhibition of Myristoylation, Membrane Binding, and CD8-ζ Association of Wild-Type Fyn by Treatment with 2-Hydroxymyristate

In an attempt to manipulate fatty acylation levels of wild-type Fyn, the effect of 2-hydroxymyristate, a potent inhibitor of NMT (25, 27) was tested. Transfected COS-1 cells expressing wild-type Fyn were treated with 2-hydroxymyristate and radiolabeled with either Tran35S-label or 125I-IC13. A nalysis of immunoprecipitated Fyn after SDS-PAGE showed that the level of 35S-labeled protein was unaffected but that the level of 125I-IC13 labeling was significantly decreased (83%) after treatment with 2-hydroxymyristate (Fig. 6 A). Subsequently, analysis of the subcellular distribution of Fyn after treatment with 2-hydroxymyristate showed a dramatic increase in the amount of soluble Fyn. More than 95% of Fyn was recovered from the membrane P100 fraction of control cells, whereas $\geq$40% was observed in the soluble fraction after 2-hydroxymyristate treatment (Fig. 6 A). Next, the effect on immunoprecipitation of wild-type Fyn with CD8-ζ was analyzed. 2-Hydroxymyristate treatment caused a significant decrease in the amount of wild-type Fyn coimmunoprecipitating with CD8-ζ (Fig. 6 B), to $\leq$40% of control levels. A fter 2-hydroxymyristate treatment, wild-type Fyn still retains glycine 2, cysteine 3, and lysines 7 and 9, all of the residues previously implicated as part of an ITAM binding motif on Fyn, yet it cannot associate with ζ.

Association of Fyn with ζ Chain: The NH2 Terminus of Fyn Alone Is Not Sufficient and Functional SH2- and SH1-kinase Domains Are Essential

To further define the nature of the observed association between Fyn and TCR ζ, the following series of experiments was performed. Fig. 7 A shows that between 10 and 15% of the total population of Fyn coimmunoprecipitated with CD8-ζ (compare lanes 7 and 11). Fyn protein was not detected in the absence of anti-CD8 antibody during immunoprecipitation (Figs. 5–7), showing that nonspecific binding of Fyn to the protein A/G + agarose beads did not occur. Fyn was only detected in anti–CD8 8 immunoprecipitates from cells expressing both CD8-ζ and Fyn (Figs. 5 A, lane 3, 6 B, and 7 A, lane 7), but not from mock transfected cells (Fig. 7 A, lane 1), cells expressing CD8-ζ alone (Figs. 5 A, lane 1, and 7 A, lane 3), or Fyn alone (Fig. 7 A, lane 5). In addition, the mixing of digitonin lysates of cells that were transfected separately with either Fyn or CD8-ζ, followed by immunoprecipitation with anti-CD8-ζ antibodies, did not result in coimmunoprecipitation of Fyn (Fig. 7 A, lane 9), highlighting that Fyn and CD8-ζ need to be present within the same cell.

Next, the functional requirements within the Fyn protein structure for association with CD8-ζ were evaluated. We first tested whether the Fyn NH2 terminus alone is sufficient for interaction with the ζ chain in cells, using Fyn(16)GFP, a chimeric construct containing the first 16 amino acids of Fyn fused to GFP. Fyn(16)GFP expressed in COS-1 cells was dually acylated with myristate and palmitate (data not shown), and after extraction with non-ionic detergent buffer, $\leq$60% of total Fyn(16)GFP was re-
covered from the detergent insoluble fraction (Fig. 7 B). Thus, Fyn(16)GFP is processed and targeted like wild-type Fyn and other chimeras containing the Fyn SH4 motif (41, 43). After coexpression of Fyn(16)GFP and CD8-ζ in COS cells, followed by digitonin lysis, no Fyn(16)GFP was detected in anti–CD8 immunoprecipitates, although there was a strong signal after immunoprecipitation with anti–GFP antibody (Fig. 7 B). Similar results were obtained with constructs containing the NH₂-terminal SH4 motif of Fyn fused to GST or β-galactosidase (data not shown). These results indicate that the NH₂-terminal SH4 motif of Fyn by itself is not sufficient for association with CD8-ζ and that additional domains of the Fyn protein are required. To identify these requirements, CD8-ζ was coexpressed with three different Fyn mutants: K₂⁹⁹M-Fyn carries a point mutation in the SH1 domain that renders Fyn kinase-inactive, ΔSH2-Fyn lacks amino acids 144-248 encoding the Fyn SH2 domain, and R₁₇₆K-Fyn contains a point mutation of a critical arginine residue in the SH2 domains of Src family kinases that reduces phosphotyrosine binding (26). Less than 15% of ΔSH2-Fyn and only ~35% of K₂⁹⁹M-Fyn and R₁₇₆K-Fyn was detected in anti–CD8 immunoprecipitates (Fig. 7 C, left, and Table III). The expression levels were similar for all constructs (Fig. 7 C, right). These findings establish that functional SH1 and SH2 domains in Fyn are important for optimal interaction with CD8-ζ.

**Association of Fyn with ζ Chain Requires Phosphorylation of ζ ITAMs by Fyn**

Based on the findings described above that kinase activity and the SH2 domain in Fyn are critical, we addressed the relevance of the ITAM motifs in the ζ chain for association with Fyn. First, the tyrosine phosphorylation status of CD8-ζ was tested by immunoprecipitation with anti–CD8 antibody followed by antiphosphotyrosine Western blotting analysis. A signal shown in Fig. 8 A, CD8-ζ was strongly tyrosine phosphorylated when coexpressed with Fyn, ΔSH2-Fyn.
Table III. Quantitative Analysis of Coimmunoprecipitation of Fyn with CD8-ζ

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative coimmunoprecipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8-ζ</td>
<td>100%</td>
</tr>
<tr>
<td>+ Fyn</td>
<td></td>
</tr>
<tr>
<td>+ K299M-Fyn</td>
<td>35%</td>
</tr>
<tr>
<td>+ ΔSH2-Fyn</td>
<td>14%</td>
</tr>
<tr>
<td>+ R176K-Fyn</td>
<td>38%</td>
</tr>
<tr>
<td>CD8-ζΔT76</td>
<td>91%</td>
</tr>
<tr>
<td>+ Fyn</td>
<td></td>
</tr>
<tr>
<td>CD8-ζ + Fyn</td>
<td>19%</td>
</tr>
<tr>
<td>(Octylglucoside)</td>
<td>14%</td>
</tr>
<tr>
<td>(Filipin)</td>
<td>54%</td>
</tr>
<tr>
<td>CD8-ζ</td>
<td>93%</td>
</tr>
<tr>
<td>+ FynKRas</td>
<td></td>
</tr>
<tr>
<td>+ G1,A,C,S-FynKRas</td>
<td>26%</td>
</tr>
</tbody>
</table>

COS-1 cells were transfected with cDNAs encoding the indicated constructs, and coimmunoprecipitation experiments were performed as described in Fig. 5. In each experiment, coimmunoprecipitation of Fyn protein construct with anti–CD8 antibody was measured in duplicate and multiple films representing different exposure times to the blot were used to ensure linearity of the signal. These values were first corrected for the total level of Fyn protein in each immunoprecipitation, as measured by the second blot were used to ensure linearity of the signal. The given 100% value of wild-type Fyn coimmunoprecipitating Fyn immunoprecipitation step (see Materials and Methods). Given numbers for each the total level of Fyn protein in each immunoprecipitation, as measured by the second autoradiography. CD8-ζ represents 10–15% of total Fyn expressed (see Fig. 7). if one main form that migrated at ~40 kD and the major radiolabeled form of CD8-ζFyn was observed to migrate at ~35 kD, as indicated by the arrows. The 40-kD band of CD8-ζT76 was tyrosine phosphorylated when coexpressed with Fyn, with moderately reduced levels compared with full length CD8-ζ, whereas no tyrosine phosphorylation was observed for the CD8-ζ4F mutant (Fig. 8 B). A analysis of the association of Fyn with the different CD8-ζ constructs showed that similar amounts (~90%) of Fyn coimmunoprecipitated with CD8-ζT76, whereas very little (~20%) Fyn was observed in the CD8-ζ4F immunoprecipitate (Fig. 8 C, and Table III). These results show that phosphorylation of ITAM tyrosines in the ζ chain by Fyn is critical for association between Fyn and ζ. A similar finding was reported by Pleiman et al. (29), who showed that the SH2 domains of Fyn and Lyn bind to the tyrosine phosphorylated ITAM in the Igα receptor.

Association of Fyn with CD8-ζ Is Stabilized in Membrane Rafts

Dual fatty acylation has previously been shown to be required for localization of Fyn to plasma membrane micro-
domains known as rafts (5, 35, 38, 41, 43). To test for the involvement of rafts in directing the interactions between Fyn and \( \zeta \) chain, two different approaches were used. First, we analyzed the sensitivity of coimmunoprecipitation of Fyn with CD8-\( \zeta \) to treatment with octylglucoside and filipin, agents that disrupt raft stability (5, 38). The association of Fyn with CD8-\( \zeta \) was almost entirely abolished by addition of octylglucoside to the digitonin lysis buffer (Table III). The interaction was also greatly reduced (~50%) by pretreatment of cells with filipin (Table III), an agent that binds cellular cholesterol, an essential component for raft integrity (5, 38). A alternate approach was based on the recent observation that, in contrast to fatty acylated proteins, farnesylated proteins are largely excluded from detergent-resistant membrane rafts (23). Fyn was tagged at the carboxy terminus with the KRas4B tail (FynKRas), which promotes farnesylation and plasma membrane targeting (15). This construct is both fatty acylated and prenylated (data not shown). A second construct containing only a functional farnesylation signal was generated by mutating the amino-terminal fatty acylation sites (G2A,C3S-FynKRas). When expressed in COS-1 cells, wild-type Fyn and the two KRas-tagged constructs were completely membrane bound (Fig. 9A). Wild-type Fyn and FynKRas were also enriched in the Triton X-100 insoluble fraction (Fig. 9B). In contrast, the amount of G2A,C3S-FynKRas as in the Triton X-100 insoluble fraction was significantly lower (Fig. 9B), suggesting that farnesylated Fyn exhibits reduced association with plasma membrane rafts. Fig. 9C and Table III show that coimmunoprecipitation of G2A,C3S-FynKRas with CD8-\( \zeta \) was significantly reduced, whereas association of FynKRas with CD8-\( \zeta \) was very similar to that of wild-type Fyn. FynKRas and G2A,C3S-FynKRas kinase activity and ability to phosphorylate \( \zeta \) ITAM tyrosines were similar to wild-type Fyn (data not shown). These observations imply that fatty acylation not only provides plasma membrane binding of Fyn, but also guides specific localization to membrane subdomains, which is essential for stabilization of the protein-protein interactions between Fyn and the \( \zeta \) chain.

**Discussion**

**Lysines 7 and 9 Are Required for Efficient N-Myristoylation of Fyn**

Lysine residues at positions 7 and 9 are conserved in...
nearly all Src family members (36, 37), and, in the context of the NH$_2$ terminus of v-Src, the lysine at position 7 is critical for efficient myristoylation (6, 19). We were therefore interested in testing the fatty acylation status of K$_{7,9}$A-Fyn, a mutant containing alanine substitutions for lysines 7 and 9. To obtain sensitive measurements of myristoylation levels, different Fyn constructs (Table I) were expressed at high levels in COS cells, and radiolabeling with the iodinated myristate analogue $^{125}$I-IC13 was performed. After immunoprecipitation and SDS-PAGE, quantitation of $^{125}$I-IC13 incorporation into Fyn was accomplished by PhosphorImaging (2), which is not feasible after radiolabeling with conventional $^3$H-labeled myristate analogues used in other studies (11). Since all Fyn constructs used in this report contain the same number of methionines and cysteines, labeling with Tran$^{35}$S-label in parallel gave precise numbers for expression levels of the protein backbone. Subsequent calculation of $^{125}$I-IC13 per unit of $^{35}$S-labeled protein provided a reliable measure of the efficiency of myristoylation for wild-type and mutant Fyn constructs.

Our results show that, as previously established for v-Src (6, 19), lysine 7 is required for efficient myristoylation of Fyn (Table I). In addition, we show that lysine 9 plays an important role in guiding effective myristoylation of Fyn (Table I). Interestingly, the effects of the individual lysine replacements add up to the total effect observed with the double K$_{7,9}$A-Fyn mutant (Fig. 1, and Table I), suggesting that each lysine residue plays an independent role. The conclusion that these lysines are directly involved in a myristoylation event is further substantiated by our finding that the K$_{7,9}$A-Fyn myristoylation defect is specifically reversed by coexpression with wild-type human NMT, but not with enzymatically impaired H$_{218}$N-NMT (28) (Figs. 3 and 4).

Reversal of the K$_{7,9}$A-Fyn myristoylation defect by additional exogenous NMT in our experiments was almost complete (Fig. 4 B). In the experiments described here, we have used cDNA encoding a 49-kD form of human NMT (28), which has full enzymatic activity in vitro and is capable of functionally complementing myristoylation deficiency in yeast (9). An additional cellular forms of NMT, with higher molecular weights (~60 kD) and different intracellular distribution patterns, have been observed (14), and recently a second mammalian NMT was characterized (13). Most notably, missing from the 49-kD form of NMT (9, 28) is an NH$_2$-terminal sequence containing a polybasic motif that is proposed to function in ribosomal targeting of NMT (14), as well as other cotranslationally active enzymes, such as N-methionylaminopeptidases (21). We have observed that neither the 60-kD form nor the NMT 1 or 2 isoforms (13) reverse the myristoylation defect in K$_{7,9}$A-Fyn more efficiently than the 49-kD form (data not shown).

Lck is the only Src family member that lacks lysines at positions 7 and 9 (36, 37) and one might question whether myristoylation of Lck is impaired relative to the other Src-related kinases. Since quantitation of fatty acylation of full length Lck proved technically difficult, we used an Lck(10)Fyn fusion construct (Table I). Our results show that the fatty acylation profile of the Lck amino terminus is very similar to that of Fyn (Table I), despite the absence of lysines 7 and 9. We infer that in Lck the serine at position 6 (Table I), a preferred residue at this site for recognition by mammalian NMT (17) is sufficient to direct efficient fatty acylation of this sequence. If myristoylation levels of full length Lck were substoichiometric, one would expect that either its membrane association would not be complete or that additional factors (e.g., CD4) would be required to guide membrane binding of Lck. However, nearly all of the Lck in cells appears to be membrane bound (18, 46). Moreover, two recent studies have shown that plasma membrane targeting of Lck can be achieved in the absence of CD4 and that the first 10 amino acids of Lck are sufficient for plasma membrane binding (4, 46). These experiments imply that myristoylation and palmitoylation of full length Lck occur efficiently.

**The NH$_2$ Terminus of Fyn Directs Fatty Acylation and Membrane Localization, but Does Not Specify an ITAM Binding Motif**

Several earlier reports have described coimmunoprecipitation of the thymic isoform of Fyn with the TCR/CD3 complex (10, 12, 33). The association was only observed under mild detergent conditions and the exact nature of the interaction remained unclear. Recently, the first 10 amino acids of Fyn were shown to be essential for specific binding of Fyn to the $\zeta$ subunit, as well as other chains of the TCR complex in heterologous systems (10). On the basis of mutagenesis experiments, it was concluded that the amino acids glycine 2, cysteine 3, and lysines 7 and 9 define a motif for binding to ITAMs and for plasma membrane localization (11).

Our results described here (Figs. 1–4), as well as earlier work from our group and others (1, 35, 41, 43), indicate that these amino acids within the Fyn SH4 domain are essential for optimal fatty acylation and membrane binding of Fyn. Using the coimmunoprecipitation of various Fyn constructs with a CD8-$\zeta$ fusion protein (16) as a model, we present three lines of evidence to demonstrate that the Fyn SH4 domain does not represent a specific ITAM binding motif.

First, we show that the reduced coimmunoprecipitation of the K$_{7,9}$A-Fyn mutant with CD8-$\zeta$ (Fig. 5, and reference 11) is specifically reversed by overexpression of NMT (Fig. 5). The effect of NMT overexpression is to increase fatty acylation and membrane binding of K$_{7,9}$A-Fyn (Figs. 3 and 4). This gain of function experiment clearly establishes that coimmunoprecipitation of Fyn with CD8-$\zeta$ does not require lysines 7 and 9 per se, but their presence is crucial for directing efficient fatty acylation. This conclusion is further substantiated by the observations made with the G$_{o,\zeta}$Fyn and Lck(10)Fyn constructs, which coimmunoprecipitate with CD8-$\zeta$ as efficiently as wild-type Fyn (Fig. 5 B). The NH$_2$-terminal sequences of G$_{o,\zeta}$Fyn and Lck(10)Fyn do not contain lysines at position 7 and 9 (Table I), but they are efficiently fatty acylated (reference 41, and Table I). Second, the loss of function experiment with 2-hydroxymyristate (Fig. 6) shows that, even in the presence of all the amino acids proposed to constitute the ITAM binding motif (glycine 2, cysteine 3, and lysines 7 and 9), coimmunoprecipitation of wild-type Fyn with CD8-$\zeta$ can be ablated. Thus, loss of association of wild-
type Fyn to CD8-ζ coincides with a reduction of fatty acylation and membrane binding (Fig. 6). Third, Fyn(16)GFP did not coimmunoprecipitate with CD8-ζ (Fig. 7 B), showing that the first 16 amino acids of Fyn are not sufficient for interaction with the ITAMs on ζ, in agreement with the results of cross-linking studies (34). This implies that other protein determinants in Fyn are involved in mediating the interactions with ζ chain. Indeed, coimmunoprecipitation of Fyn was observed to require both a functional kinase as well as an SH2 domain (Fig. 7 C).

**Colocalization of Fyn and ζ Chain in Cell Surface Rafts**

Coimmunoprecipitation of Fyn with TCR subunits occurs only under mild detergent conditions (10, 12, 33), which has been taken as evidence for low affinity protein–protein interactions. Our findings with Fyn, and a recently growing interest in membrane microdomains (5, 38), suggest the involvement of additional mechanisms for stabilization of the interactions between Src kinases and TCR subunits, which is supported by several recent independent reports (24, 44, 45). In biological membranes, especially plasma membranes, phase-separated “rafts” exist, representing lipid domains enriched in cholesterol and sphingolipids that provide scaffolds for clustering of transmembrane and peripherally associated proteins (5, 38). These rafts or microdomains can be recovered from cells using mild detergent conditions (5). There is growing evidence that key components of TCR-mediated signaling events are localized to rafts. For example, palmitoylation of the T cell transmembrane protein LAT is essential for localization in rafts and T cell tyrosine phosphorylation events (45). Src family kinases, especially Fyn and Lck, are constitutively localized in rafts via dual fatty acylation motifs (35, 41, 43). A citivation of the TCR results in specific accumulation of ζ chains in plasma membrane rafts (24). In COS cells, ~20% of CD8-ζ is in the detergent-insoluble fraction (data not shown), similar to the distribution of the ζ chain in resting T cells (7), and this may explain in part why only a limited amount (~20%) of total Fyn coimmunoprecipitates with CD8-ζ in COS cells. The functional significance of raft localization is evidenced by the finding that disruption of cell surface rafts interferes with early T cell signaling events (44). These observations establish a connection between local accumulation of signaling molecules in plasma membrane rafts and regulated T cell signaling events.

Based on the results reported here and by others (35, 39, 41, 43), the following model emerges. M yristoylation and palmitoylation within the NH₂-terminal SH4 domain directs Fyn into detergent-resistant plasma membrane rafts and positions the downstream SH2 and kinase domains in close proximity to the TCR ζ chain. Phosphorylation of the ζ ITAMs by Fyn (Fig. 8) allows subsequent interaction between the phosphorylated ITAMs and the Fyn SH2 domain (Figs. 7 and 8). A accumulation of Fyn and the ζ chain in rafts increases the local concentration of both proteins and drives their association. The stability and the extent of Fyn SH2/ζ phosphotyrosine interaction is apparently dependent on the integrity of the rafts since treatment with octylglucoside and filipin, agents that disrupt rafts (5, 38), results in dissociation of the Fyn/ζ complex (Table III), presumably due to a decrease of the effective local protein concentrations. This implies that the Fyn SH2/ζ phosphotyrosine interaction is relatively weak, as found for several other SH2/phosphotyrosine interactions (20). In T cells, dissociation of the Fyn SH2 domain from the ζ chain would make ITAM M phosphorytrosine residues on ζ accessible for binding to the SH2 domains of other signaling molecules, such as ZAP-70. This model explains why undermyristoylated and underpalmitoylated forms of Fyn, that are not efficiently membrane bound, as well as a farnesylated Fyn construct that is not enriched in membrane subdomains (Fig. 9), do not interact well with the ζ chain. These studies illustrate that the role of the NH₂-terminal SH4 motif is to properly position Src kinases within specific locations of cellular membranes for optimal interaction of downstream Src homology domains with other membrane-bound signaling molecules.

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