Multiple Features of the p59^{fyn} src Homology 4 Domain Define a Motif for Immune-Receptor Tyrosine-based Activation Motif (ITAM) Binding and for Plasma Membrane Localization

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Abstract. The src family tyrosine kinase p59^{fyn} binds to a signaling motif contained in subunits of the TCR known as the immune-receptor tyrosine-based activation motif (ITAM). This is a specific property of p59^{fyn} because two related src family kinases, p60^{src} and p56^{lck}, do not bind to ITAMs. In this study, we identify the residues of p59^{fyn} that are required for binding to ITAMs. We previously demonstrated that the first 10 residues of p59^{fyn} direct its association with the ITAM. Because this region of src family kinases also directs their fatty acylation and membrane association (Resh, M.D. 1993. *Biochim. Biophys. Acta.* 1155:307–322; Resh, M.D. 1994. *Cell.* 76:411–413), we determined whether fatty acylation and membrane association of p59^{fyn} correlates with its ability to bind ITAMs. Four residues (Gly2, Cys3, Lys7, and Lys9) were required for efficient binding of p59^{fyn} to the TCR. Interestingly, the same four residues are present in p56^{lyn}, the other src family tyrosine kinase known to bind to the ITAM, suggesting that this set of residues constitutes an ITAM recognition motif. These residues were also required for efficient fatty acylation (myristoylation at Gly2 and palmitoylation at Cys3), and plasma membrane targeting of p59^{fyn}. Thus, the signals that direct p59^{fyn} fatty acylation and plasma membrane targeting also direct its specific ability to bind to TCR proteins.

THE phosphorylation of proteins on tyrosine residues is an early event that is required for TCR signal transduction (June et al., 1990; Mustelin et al., 1990). Because the proteins within the TCR complex do not contain intrinsic enzymatic activity, studies have focused on the associated protein tyrosine kinases that are activated by TCR engagement. Two members of the src family of protein tyrosine kinases, p59^{fyn} and p56^{lck}, are important for TCR signaling and have been localized to the receptor complex (reviewed in Samelson and Klausner, 1992; Malissen and Schmitt-Verhulst, 1993; Weiss and Littman, 1994; Howe and Weiss, 1995). p59^{fyn} is directly associated with the TCR subunits by binding to the signaling motif known as the immune-receptor tyrosine-based activation motif (ITAM)[1] (Samelson et al., 1990; Gassmann et al., 1992; Gauen et al., 1992, 1994), whereas p56^{lck} is brought into the complex upon antigen recognition by its association with the CD4 and CD8 coreceptor proteins (Rudd et al., 1988; Veillette et al., 1988; Mittler et al., 1989; Shaw et al., 1989; Collins et al., 1992; Dianzani et al., 1992).

We have been interested in defining the structural features that mediate these interactions. Sequence comparison of src family members demonstrates that they have a modular architecture with three highly conserved src homology (SH) domains that have been designated as the SH1, SH2, and SH3 domains. These domains are responsible for enzymatic activity and mediate protein–protein interactions. An additional nonconserved domain that is contained in the amino-terminal 40–70 residues of each kinase is unique to each family member. Specific and specialized functions of each kinase are thought to be mediated by this domain. In fact, the specific interactions of p56^{lck} with CD4 and CD8 and between p59^{fyn} and TCR subunits are mediated by these unique domains. In the case of p56^{lck}, a Cys-X-X-Cys motif (residues 23–26) is required for binding to a similar Cys-X-Cys sequence in CD4 and CD8 (Shaw et al., 1990; Turner et al., 1990). For p59^{fyn}, however, the sequences responsible for specific binding to TCR subunits are contained within the first 10 residues (Gauen et al., 1992). Because this amino-terminal region regulates fatty acylation and membrane association of src family kinases, Resh (1993) has recently proposed its designation as the SH4 domain.

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1. *Abbreviations used in this paper*: ITAM, immune-receptor tyrosine-based activation motif; SH, src homology; VSV G, vesicular stomatitis virus glycoprotein.

In this study, we identify the features of the p59^{fyn} SH4 domain that mediate its association with TCR subunits. Site-directed mutagenesis was used to generate a panel of p59^{fyn} proteins that contained alanine substitutions within the SH4 domain. Because this domain is responsible for fatty acylation and membrane localization of src family kinases, we also determined whether the fatty acylation and membrane localization of each mutated p59^{fyn} protein correlated with its ability to bind to TCR subunits. Our results demonstrate that multiple features of the SH4 domain are responsible for mediating the specific interaction of p59^{fyn} with the TCR and plasma membranes.

Materials and Methods

DNA Constructs and Mutagenesis

Chimeric TCR DNA constructs that contain sequences encoding the extracellular domain of the vesicular stomatitis virus glycoprotein (VSV G) and the cytoplasmic domain of the TCR or ζ chains (designated as Gε and Gζ, respectively) were described previously (Gauen et al., 1992). The following DNA constructs were also previously described: p59^{fyn}, which encodes the hematopoietic-specific isoform of mouse p59^{fyn}, fyn/myc, which encodes a myc epitope–tagged form of p59^{fyn}, and src10/fyn/myc, which encodes a myc epitope–tagged form of a p59^{fyn} protein in which the first 10 amino acids of chicken p60$^{c\text{-}src}$ were substituted for that of p59^{fyn} (Gauen et al., 1992). The DNA constructs encoding the p59^{fyn} proteins that contain individual alanine substitutions within the first 10 amino acids (depicted in Fig. 1 *B*) were created by site-directed mutagenesis of the fyn/myc DNA construct. Inverse PCR (Hemsley et al., 1989) were performed using the following oligonucleotides (mutated codons are underlined) with the reverse primer 5′CCGGTACCCAATTCGCCCTA (which anneals with pBluescript [Stratagene]): 5′CGAATTCATAATGGCCTGTGTGCAATGTAA (G2A), 5′CGAATTCATAATGGGCGCTGTGCAATGTAAGGA (C3A), 5′CGAATTCATAATGGGCTGTGCGCAATGTAAGGATAA (V4A), 5′CGAATTCATAATGGGCTGTGTGGCATGTAAGGATAAAGA (Q5A), 5′CGAATTCATAATGGGCTGTGTGCAAGCTAAGGATAAAGAAGC (C6A), 5′CGAATTCA TAATGGGCTGTGTGCAATGTGCGGATAAAGAAGCAGC (K7A), 5′CGAATTCATAATGGGCTGTGTGCAATGTAAGGCTAAAGAAGCAGCGAA (D8A), 5′CGAATTCATAATGGGCTGTGTGCAATGTAAGGATGCAGAAGCAGCGAAACT (K9A), 5′CGAATTCATAATGGGCTGTGTGCAATGTAAGGATAAAGCAGCAGCGAAACTGAC (E10A). The src10/Cys3 DNA construct was generated by PCR using the src10/fyn/myc plasmid as a template with the oligonucleotides 5′ACCGCTCGAGACCATGGGCTGCAGCAAGAGCAAGCCC (encoding the amino acids MGCSKSKP) and 5′GGAAACAGCTATGACCATGAT (which binds to pBluescript). The resulting DNA fragment was digested with XhoI and EcoRI and ligated into pBluescript SK$^+$. The K7,9A fyn construct was made by PCR using the p59^{fyn} plasmid as a template and the oligonucleotides: 5′GCAGATGCTGAAGCAGCGAAACTG and 5′ACATTGCACACAGCCCATTATCCA. The fyn Y531F construct was generated by PCR using the p59^{fyn} plasmid as a template and the oligonucleotides 5′TTAACTCGAGTCACAGGTTTTCACCGGGCTGAAACTGGGG and 5′TTGTAATACGACTCACTATAG (which binds to pBluescript). DNA sequence analysis using Sequenase (Amersham, Sanger et al., 1977) was performed to verify the sequences of the constructs.

DNA Transfections, Immunoprecipitations, In Vitro Kinase Reactions, and Binding Assays

HeLa cells were cotransfected with p59^{fyn} and VSV G/TCR chimeric cDNA constructs in a transient expression system. This expression system uses a recombinant vaccinia virus encoding a bacteriophage T7 RNA polymerase (Fuerst et al., 1986) to infect cells, followed by liposomal-mediated transfection of cDNAs. TCR-binding assays were performed by coimmunoprecipitation of p59^{fyn} with VSV G/TCR chimeric proteins using an mAb that recognizes VSV G (I1) (Lefrancois and Lyles, 1982). Binding of p59^{fyn} was detected by in vitro kinase reactions of the VSV G immunoprecipitates. These procedures were performed as previously described (Gauen et al., 1992).

Immunoblotting

Immunoblotting was performed for the binding assays on lysates from duplicate cultures of cells as previously described (Gauen et al., 1992), using the antibodies and developing reagents described below. VSV (antiserum purchased from Lee Biomolecular Research, Inc.) and p59^{fyn} (antiserum kindly provided by André Veillette) immunoblots were performed using rabbit polyclonal antisera. Myc epitope immunoblots were performed on cell lysates using the 9E10 mAb (Evan and Bishop, 1985). Affinity-purified HRP-conjugated goat anti–rabbit or goat anti–mouse secondary antibodies (Cappel Laboratories, Durham, NC) were used. Proteins were detected by chemiluminescence (DuPont/NEN, Boston, MA). When immunoblotting immunoprecipitates prepared with the p59^{fyn} antiserum, biotinylated 9E10 antibodies and HRP-conjugated streptavidin were used.

Cellular Fractionation

HeLa cells were transfected with the indicated DNA constructs and analyzed 4–6 h later. The cells were scraped from the dish, pelleted, resuspended in hypotonic buffer (20 mM Tris, pH 6.8, 1 mM $MgCl_2$, 5 mM KCl) containing the protease inhibitor aprotinin (20 μg/ml; Sigma Immunochemicals, St. Louis, MO), and broken using 25 strokes with a tight-fitting pestle in a Dounce homogenizer. The suspension was centrifuged at 1,000 *g* to remove unbroken cells and nuclei, layered over a 30% sucrose cushion, and spun for 30 min at 200,000 *g*. The supernatants and pellets were adjusted to equivalent volumes, loaded onto 8% SDS-polyacrylamide gels, and separated by electrophoresis. The proteins were transferred to nitrocellulose and immunoblotted using polyclonal antiserum to p59^{fyn}.

Immunofluorescent Microscopy

HeLa cells were cultured on glass coverslips and transfected with the indicated DNA constructs. 5–6 h after transfection, the cells were fixed with 3% paraformaldehyde and permeabilized with 1% NP-40 (Sigma). mAbs to p59^{fyn} (1S; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and Zymed Laboratories, Inc., South San Francisco, CA) or to the myc epitope (9E10) were incubated with the cells for 20 min at room temperature. The cells were washed in PBS (1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 138 mM NaCl, 2.7 mM KCl) with 25 mM glycine and incubated for 20 min at room temperature with an FITC-conjugated goat anti–mouse secondary antibody (U.S. Biochemical Corp., Cleveland, OH). The fluorescent staining was visualized using a confocal microscope (Bio Rad Laboratories, Hercules, CA).

^{3}H-Fatty Acid Labelings

HeLa cells were transfected with the indicated DNA constructs in triplicate. 5–6 h later, cells were incubated in media containing 100–300 mCi/ml of [9,10-^{3}H]myristate (33.5 Ci/mmol) or [9,10-^{3}H]palmitate (60 Ci/mmol) for 1–2 h at 37°C. The cells were lysed in a detergent buffer containing 1% NP-40, 0.4% CHAPS (Pierce Chemical Co., Rockford, IL), 1% sodium deoxycholate (Sigma), 25 mM Tris, pH 6.8, 25 mM NaF, 150 mM NaCl, 100 mM sodium orthovanadate, and the protease inhibitor aprotinin (20 μg/ml; Sigma). Immunoprecipitates were prepared from postnuclear supernatants using a polyclonal antiserum to p59^{fyn}. The ^{3}H-labeled proteins were detected by fluorography of SDS-polyacrylamide gels treated with 2,5 diphenyl-oxazole (PPO).

^{3}H-Fatty Acid Analysis

Radioactive fatty acids liberated by alkaline hydrolysis of the p59^{fyn} proteins were analyzed as described (Linder et al., 1993) with minor modifications. Proteins in a polyacrylamide gel slice were hydrolyzed with 1.5 M NaOH, and fatty acids were extracted with chloroform/methanol. After the addition of 50 mg of palmitic acid as a carrier, extracted fatty acids were analyzed by HPLC on a Beckman; Ultrasphere C_{18} reversed-phase column with acetonitrile/0.1% trifluoroacetic acid (80:20) (Beckman Instrs., Palo Alto, CA). Fatty acids were also analyzed by TLC on C_{18} reversed-phase plates (Whatman, Clifton, NJ) with acetonitrile/acetic acid (90:10) as the mobile phase.

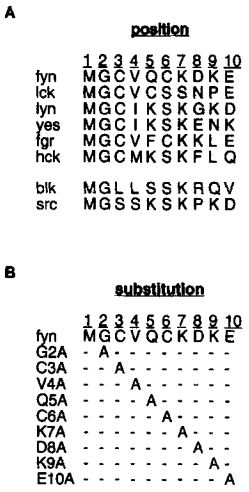

Figure 1. Analysis of the amino-terminal residues of p59^{fyn}. (*A*) Comparison of the amino-terminal residues of src family kinases. (*B*) Diagram of p59^{fyn} proteins containing single amino acid substitutions. The amino-terminal residues of p59^{fyn} are indicated. The position of each amino acid is designated numerically from the initiator methionine. A series of nine p59^{fyn} proteins was generated, each of which contains an alanine substitution at the indicated position. The mutated proteins are designated by the amino acid present in p59^{fyn}, the position of the residue, and the amino acid substituted. Amino acids are indicated using the single-letter code.

Results

Determination of Critical Residues of p59^{fyn} for Binding to TCR Proteins

We previously demonstrated that p59^{fyn} associates with multiple TCR subunits. All these subunits contain a signaling motif known as the ITAM, and we showed that p59^{fyn} interacts directly with the ITAM (Gauen et al., 1994). This interaction is specific for p59^{fyn} because two highly related src family kinases, p56^{lck} and p60^{src}, did not bind to the chimeric TCR proteins. Mapping studies revealed that a small region of p59^{fyn} known as the SH4 domain is responsible for this binding (Gauen et al., 1992). This was surprising because a comparison of this region between other src family kinases (Resh, 1993) demonstrates that many of these residues are shared (Fig. 1 *A*). We were therefore interested in determining the unique features of the p59^{fyn} SH4 domain that are responsible for its ability to bind to TCR proteins.

To investigate which amino acids are responsible for binding specificity, nine mutated p59^{fyn} proteins containing single alanine substitutions were generated (depicted in Fig. 1 *B*). These proteins were tagged at the carboxy terminus with a myc epitope that is recognized by the 9E10 mAb (Evan and Bishop, 1985). This allowed immunoprecipitation and immunoblotting of the mutated proteins without recognizing endogenous p59^{fyn}. All of the mutated p59^{fyn} proteins demonstrated apparent wild-type levels of kinase activity (data not shown). Each of the proteins was then tested for its ability to associate with chimeric TCR proteins after transient expression in HeLa cells. For these experiments, we used a chimeric TCR protein, Gε, which contains the extracellular domain of the VSV G fused to the cytoplasmic domain of CD3ε. Association of p59^{fyn} with the chimeric TCR protein was assessed by measuring the presence of kinase activity in VSV G immunoprecipitates in vitro. As reported previously, in vitro kinase reactions from cells coexpressing p59^{fyn} and Gε result in phosphorylated proteins migrating with apparent molecular mobilities of 75 and 65 kD, representing phosphorylated Gε and p59^{fyn}, respectively (Gauen et al., 1992, and Fig. 2 *A*, lane *1*). We also reported previously that this binding is

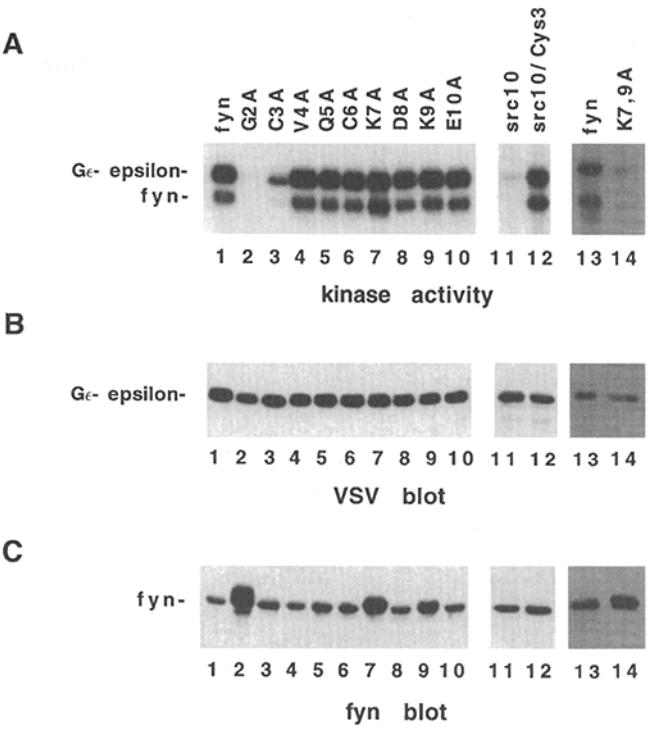

Figure 2. Association of mutated p59^{fyn} proteins with Gε. (*A*) In vitro kinase reactions of VSV G immunoprecipitations. HeLa cells were cotransfected with Gε and the indicated p59^{fyn} constructs (lanes *1–14*). VSV G immunoprecipitates were prepared from the cell lysates using the I1 mAb. Coprecipitating kinase activity was detected by in vitro kinase reactions. The ^{32}P-labeled proteins were electrophoretically separated on SDS-polyacrylamide gels and detected by autoradiography. (*B* and *C*) VSV and p59^{fyn} or myc epitope immunoblots. Lysates were prepared from duplicate cultures of HeLa cells as indicated in *A*. Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were incubated with a rabbit polyclonal antiserum to VSV (*B*), a rabbit polyclonal antiserum to p59^{fyn} (*C*, lanes *1–10* and *13–14*), or an mAb to the myc epitope tag (*C*, lanes *11* and *12*). The filters were washed and incubated with an HRP-conjugated goat anti–rabbit antibody and developed using chemiluminescence reagents. The positions of the Gε (75 kD) and p59^{fyn} (65 kD) proteins are indicated.

not caused by nonspecific interactions with the VSV G antibody and that it requires the presence of the ITAM signaling motif in the cytoplasmic domain of Gε (Gauen et al., 1992, 1994). Little or no kinase activity was detected in the VSV G immunoprecipitates from cells that coexpress Gε with the G2A or C3A proteins (Fig. 2 *A*, lanes *2* and *3*). The remainder of the p59^{fyn} proteins retained the ability to bind Gε (Fig. 2 *A*, lanes *4–10*). Identical results were obtained when a fusion protein containing the cytoplasmic domain of another TCR subunit, Gζ (Gauen et al., 1992), was used (data not shown). Immunoblotting of whole-cell lysates with antibodies to VSV and p59^{fyn} demonstrated that all of the proteins were highly expressed (Fig. 2, *B* and *C*, lanes *1–10*), confirming that the inability of G2A and C3A to associate with Gε was not caused by lower levels of expression. We were concerned that the detection of K7A kinase activity coprecipitating with Gε was caused by higher levels of K7A expression. Even when K7A was ex-

pressed at levels lower than p59^{fyn}, however, coprecipitating kinase activity was observed (data not shown). These data confirmed that K7A can bind Gε as efficiently as p59^{fyn}. Therefore, only Gly2 and Cys3 appeared to be the critical residues for binding.

Basic Residues and Gly2 and Cys3 within the SH4 Domain Provide the Specificity for p59^{fyn} Binding to the ITAM

Our data pointed to two residues of the p59^{fyn} SH4 domain, Gly2 and Cys3, as the critical residues for binding. p56^{lck}, which also contains these two residues, however, does not bind directly to the TCR. Comparisons of the SH4 domains of src family kinases (Fig. 1 *A*) show that key features of the p59^{fyn} SH4 domain are lysines at positions 7 and 9 and a cysteine residue at position 3. In contrast, the p56^{lck} SH4 domain does not contain any lysine residues and the SH4 domain of p60^{src} (which also cannot bind the TCR) lacks Cys3. Therefore, it was possible that the combination of Cys3 in the presence of Lys7 and Lys9 was responsible for the unique ability of the p59^{fyn} SH4 domain to bind to TCR subunits. To test this hypothesis, we substituted a cysteine residue for Ser3 in the src10/fyn protein, where the SH4 domain of p60^{src} had been substituted for that of p59^{fyn}. The src10/Cys3 protein was able to associate with Gε as efficiently as p59^{fyn}, whereas src10/Ser3 was unable to associate (Fig. 2 *A*, lanes *11* and *12* and data not shown). Immunoblotting confirmed that all of the proteins were similarly expressed (Fig. 2, *B* and *C*, lanes *11* and *12*). To test whether the presence of dual lysines at positions 7 and 9 were also critical for binding, we substituted alanines at both positions in p59^{fyn} (K7,9A). Although K7,9A was expressed at comparable levels to p59^{fyn} (Fig. 2 *C*, lanes *13* and *14*), the loss of both lysines greatly reduced binding to Gε (Fig. 2 *A*, lanes *13* and *14*). Therefore, the combination of Cys3 with Lys7 and Lys9 in the SH4 domain of p59^{fyn} is responsible for its unique ability to direct binding to TCR subunits. These data suggest that the failure of p56^{lck} to bind TCR proteins is caused by the lack of two lysine residues in its SH4 domain.

Fatty Acylation of p59^{fyn} Proteins

All src family kinases are N-myristoylated at Gly2, and with the exception of p55^{blk} and p60^{src}, may be palmitoylated at neighboring cysteine residues (Paige et al., 1993; Koegl et al., 1994; Resh, 1994). The observation that residues of p59^{fyn} critical for binding to chimeric TCR proteins are potential sites for fatty acylation led us to characterize the acylation status of the panel of mutated p59^{fyn} proteins. To confirm that the myc epitope–tagged p59^{fyn} expressed in our system was palmitoylated, HeLa cells expressing this protein were incubated with radioactive palmitate. Radioactivity derived from [^{3}H]palmitate was incorporated into epitope-tagged p59^{fyn}, but not into epitope-tagged src10/fyn (Fig. 3 *A*, lanes *1* and *2*), which lacks cysteine residues within the SH4 domain and is not palmitoylated (Alland et al., 1994; Shenoy-Scaria et al., 1994). Immunoblotting of whole-cell lysates confirmed that both proteins were expressed at high levels (Fig. 3 *A*, lanes *3* and *4*). These results suggest that the epitope-tagged p59^{fyn} expressed in our system is palmitoylated.

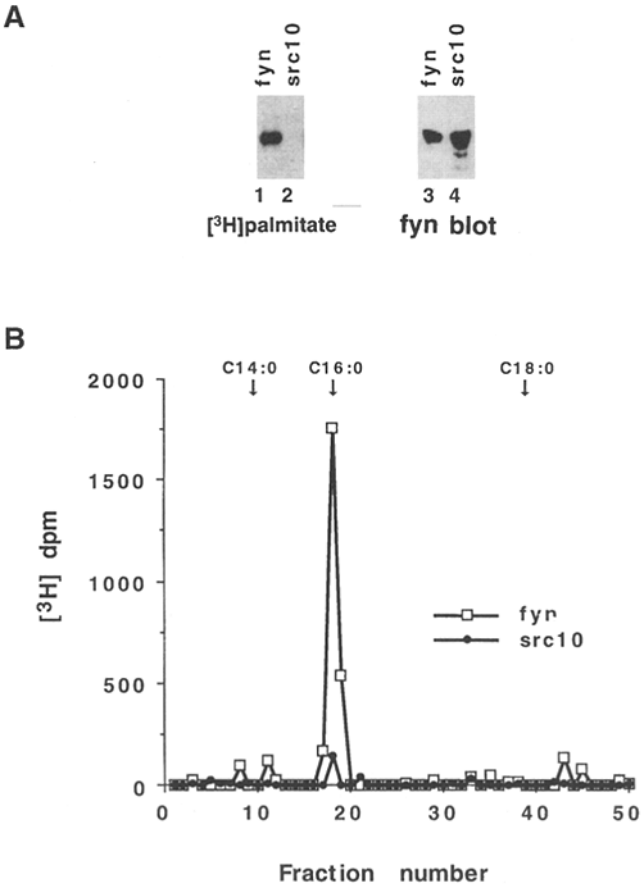

Figure 3. Incorporation of [^{3}H]palmitate into p59^{fyn} proteins in HeLa cells. (*A*) HeLa cells transfected with a cDNA encoding myc epitope–tagged p59^{fyn} (lanes *1* and *3*) or myc epitope–tagged src10/fyn (lanes *2* and *4*) were incubated with [^{3}H]palmitate. p59^{fyn} immunoprecipitates were prepared from cell lysates and separated by SDS-PAGE. [^{3}H]Palmitate incorporation was detected by fluorography (lanes *1* and *2*). A portion of each lysate was analyzed by immunoblotting with antisera to p59^{fyn} (lanes *3* and *4*), as described in Fig. 2 *C*. (*B*) Reversed-phase chromatography of fatty acids hydrolyzed from radiolabeled myc epitope-tagged p59^{fyn} and src10/fyn. Immunoprecipitates of p59^{fyn} proteins from transfected HeLa cells were resolved by SDS-PAGE. Gel slices containing p59^{fyn} or src10/fyn were excised and subjected to base hydrolysis. Extracts of the hydrolysates were chromatographed over a C_{18} reversed-phase column, and radioactive fatty acids were detected by scintillation counting. Elution profiles of the p59^{fyn} extracts (*open squares*) and the src10/fyn extracts (*closed circles*) are depicted; the positions of the myristate (C14:0), palmitate (C16:0), and stearate (C18:0) standards are indicated by arrows.

To establish that the radioactivity incorporated into the protein was authentic thioester-linked palmitate, radioactive fatty acids were released from the proteins by alkaline hydrolysis and analyzed by HPLC. Almost all the radioactivity extracted from p59^{fyn} (Fig. 3 *B*, *open squares*) comigrated with the palmitate standard (Fig. 3 *B*, fractions *19–21*). Base hydrolysates resolved by reversed-phase TLC gave identical results (data not shown). No significant radioactivity was extracted from src10/fyn (Fig. 3 *B*, *closed circles*). Thus, noncovalently associated radioactivity was not carried through the immunoprecipitation and extraction pro-

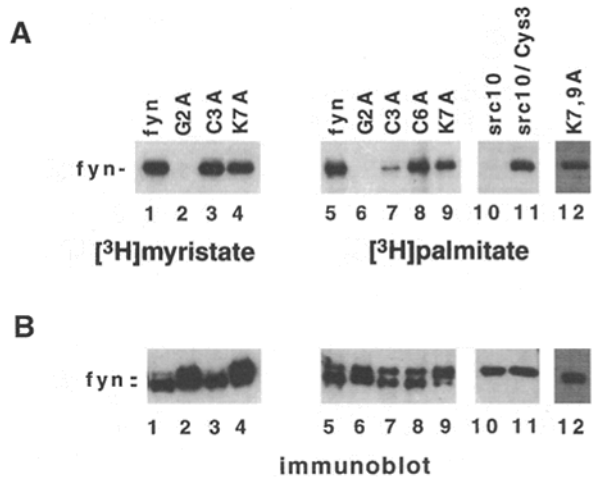

Figure 4. ^{3}H-fatty acid labeling of p59^{fyn} proteins. (*A*) [^{3}H]Myristate and [^{3}H]palmitate labeling of myc epitope–tagged p59^{fyn} proteins. HeLa cells were transfected with the indicated DNA constructs and incubated with [^{3}H]myristate (lanes *1–4*) or [^{3}H]palmitate (lanes *5–12*). The incorporated radioactivity was detected in p59^{fyn} immunoprecipitates by SDS-PAGE and fluorography. (*B*) Immunoblotting of p59^{fyn} immunoprecipitates from *A*. A portion of each immunoprecipitate from the [^{3}H]myristate-labeled cells (lanes *1–4*) or from the [^{3}H]palmitate-labeled cells (lanes *5–12*) was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose filters and incubated with a biotinylated mAb (9E10) to the myc epitope tag (lanes *1–11*) or with p59^{fyn} antisera (lane *12*). The filters were developed using HRP-conjugated streptavidin (lanes *1–11*) or HRP-conjugated goat anti–rabbit antibodies (lane *12*) and chemiluminescence reagents.

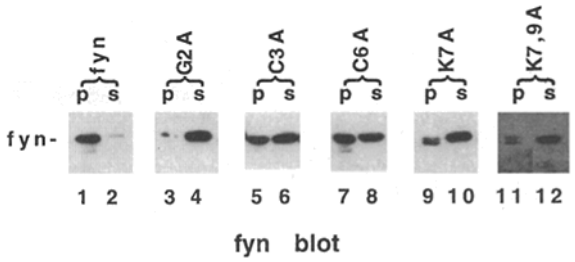

Figure 5. Fractionation of HeLa cells expressing p59^{fyn} proteins. HeLa cells were transfected with the indicated p59^{fyn} constructs and homogenized in hypotonic buffer. The postnuclear supernatants were layered over a sucrose cushion and centrifuged at 200,000 *g* for 30 min. The pellet (*p*) and supernatant (*s*) fractions were adjusted to equivalent volumes. Equal portions of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with antiserum to p59^{fyn}. The positions of the p59^{fyn} proteins are indicated. The results shown are representative of six independent experiments.

cedures. These data demonstrate that the radioactivity incorporated into p59^{fyn} was in fact palmitate and did not arise from metabolically interconverted myristate.

To determine whether there was a correlation between fatty acylation and the ability to bind TCR subunits, the mutated p59^{fyn} proteins were tested for metabolic incorporation of [^{3}H]myristate and [^{3}H]palmitate (Fig. 4 *A*, lanes *1–4* and *5–12*, respectively). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. As expected, p59^{fyn} and C3A were labeled after [^{3}H]myristate incubation, whereas no labeling was observed for G2A, which lacks the site of myristate attachment (Fig. 4 *A*, lanes *1–3*). K7A also incorporated [^{3}H]myristate, suggesting that unlike for p60$^{v\text{-}src}$ (Kaplan et al., 1988), Lys7 is not absolutely required for myristoylation of p59^{fyn} (Fig. 4 *A*, lane *4*). Although immunoblotting of the immunoprecipitates demonstrated that all proteins were efficiently immunoprecipitated, K7A was expressed at higher levels than p59^{fyn}. Because the amount of [^{3}H]myristate incorporated into K7A was equal to or less than wild-type p59^{fyn} (compare Fig. 4 *A*, lanes *1* and *4* , and *B*, lanes *1* and *4*), myristoylation of K7A is not as efficient as the wild type. Thus, the presence of Lys7 may enhance the ability of p59^{fyn} to be myristoylated.

Metabolic incorporation of [^{3}H]palmitate revealed efficient labeling of the p59^{fyn}, C6A, K7A, src10/cys3, and K7,9A proteins, with no labeling of the G2A protein (Fig. 4 *A*, lanes *5–12*). Greatly reduced labeling of the C3A protein was observed (Fig. 4 *A*, lane *7*), suggesting that although Cys3 is the primary site of p59^{fyn} palmitoylation, Cys6 is also palmitoylated to a low degree. HPLC analysis of fatty acids hydrolyzed from C3A confirmed that the radioactivity incorporated into the protein was thioester-linked [^{3}H]palmitate (data not shown). Since all the p59^{fyn} proteins that can bind the TCR were labeled with radioactive palmitate, palmitoylation is probably required for binding. It is not sufficient by itself because K7,9A, which labeled with radioactive palmitate, is not capable of binding to the TCR.

Membrane Stability and Subcellular Localization of p59^{fyn} Proteins

To determine if the ability of the mutated p59^{fyn} proteins to bind TCR proteins correlated with their ability to associate with membranes, the membrane stability of p59^{fyn} and the p59^{fyn} proteins containing substitutions within the SH4 domain were studied using cellular fractionation. Crude membrane and cytoplasmic fractions were prepared from HeLa cells that expressed each protein. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. As expected, most of the p59^{fyn} protein (70–90%) was recovered in the membrane fraction (Fig. 5, lanes *1* and *2*), and most of the G2A protein was recovered in the cytoplasmic fraction (80–90%, Fig. 5, lanes *3* and *4*). The presence of the myc epitope tag or alanine substitutions in the V4A, Q5A, D8A, and K9A proteins did not alter the percentage of p59^{fyn} molecules that were recovered in the membrane fraction (data not shown). Interestingly, the K7,9A protein was primarily in the cytoplasmic fraction, with ~10–20% of the protein located in the membrane fraction (Fig. 5, lanes *11* and *12*). The C3A, C6A, and K7A proteins showed a more modest redistribution, with ~40–50, 50–60, and 20–30% of the protein located in the membrane fraction, respectively (Fig. 5, lanes *5–10*). Thus, the membrane stability of the mutated p59^{fyn} proteins did not absolutely reflect their ability to bind TCR subunits.

It was possible that the restribution of some of the mutated proteins to the cytosolic fraction was an artifact of

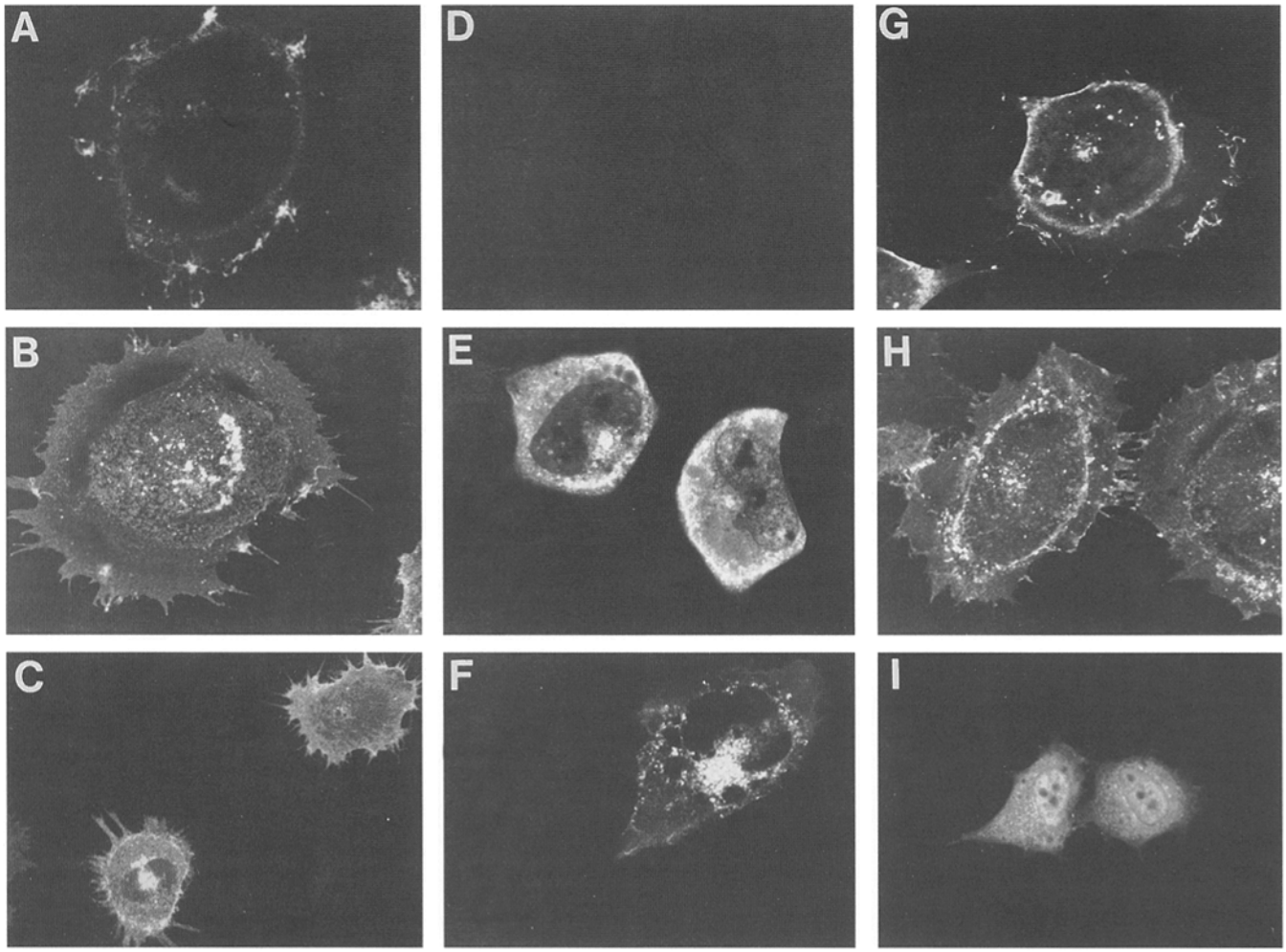

Figure 6. Confocal microscopy of HeLa cells expressing p59^{fyn} proteins. HeLa cells were transfected with the p59^{fyn} (*A*), the myc epitope–tagged p59^{fyn} (*B*), fynY531F (*C*), mock-transfected (*D*), G2A (*E*), C3A (*F*), C6A (*G*), K7A (*H*), or K7,9A (*I*) DNA constructs. The cells were fixed in 3% paraformaldehyde and permeabilized. Cells were then incubated with an mAb antibody to p59^{fyn} (1S; *A*, *C*, and *I*), or to the myc epitope tag (9E10; *B*, *D*, and *E–H*), followed by a fluorescein-conjugated goat anti–mouse secondary antibody. Immunofluorescent staining was visualized using confocal microscopy. The images shown are typical of the staining pattern seen in transfected cells from four independent experiments. No differences were observed for cells expressing the myc epitope–tagged p59^{fyn} protein when stained with the 1S or 9E10 antibodies (data not shown).

the cell fractionation procedure. Thus, to confirm the subcellular localization of the mutated proteins, confocal microscopy was performed. Cells expressing each protein were fixed with 3% paraformaldehyde, permeabilized, and stained with mAbs to p59^{fyn} or to the myc epitope tag. Both p59^{fyn} and the epitope-tagged p59^{fyn} protein demonstrated plasma membrane and bright, punctate staining throughout the cell (Fig. 6, *A* and *B*), demonstrating that the epitope tag did not alter the subcellular localization of p59^{fyn}. As an additional control, a constitutively active form of p59^{fyn} in which the regulatory tyrosine at position 531 was changed to a phenylalanine was also examined. This protein demonstrated similar staining to both p59^{fyn} and the epitope-tagged p59^{fyn} protein (Fig. 6 *C*). The V4A, Q5A, D8A, and K9A proteins were localized similarly to wild-type p59^{fyn} (data not shown), whereas mock-transfected cells demonstrated no staining for either antibody (Fig. 6 *D*; data not shown). Examination of cells expressing G2A or K7,9A showed no plasma membrane staining, with only cytoplasmic and nuclear staining (Fig. 6, *E* and *I*). Minimal plasma membrane staining was observed for cells expressing C3A. Instead, punctate and bright perinuclear staining was apparent (Fig. 6 *F*). The lack of Cys3 apparently causes a significant reduction in plasma membrane localization with a relocalization of p59^{fyn} to intracellular membranes. Therefore, the C3A protein detected in the membrane pellets by cellular fractionation (Fig. 5, lane *5*) was probably associated with intracellular membranes. In contrast, C6A and K7A, which both had reduced membrane association by cellular fractionation, demonstrated clear plasma membrane staining in addition to perinuclear staining (Fig. 6, *G* and *H*). This suggests that mutations of the cysteine at position 6 or the lysine at position 7 have reduced the membrane stability of p59^{fyn}, but did not completely abrogate plasma membrane targeting. Both fatty acylation and basic residues are therefore important for plasma membrane targeting of p59^{fyn}. Furthermore, only mutations of residues that were critical for binding of

p59^{fyn} to TCR proteins (Gly2, Cys3, and Lys7 + Lys9) resulted in a loss of plasma membrane targeting. Thus, a combination of Gly2, Cys3, and basic residues in the p59^{fyn} SH4 domain directs its efficient targeting to the plasma membrane and allows its binding to the TCR.

Discussion

Critical in the initiation of antigen-receptor signal transduction is the phosphorylation of a conserved signaling motif known as the ITAM that is found in the conserved components of the T cell antigen receptor, the B cell antigen receptor, the high affinity IgE receptor, and the low affinity IgG receptor, CD16. Although the exact mechanism is not known, phosphorylation of the ITAM is thought to be mediated by src family kinases, either directly associated with the ITAM or recruited into the receptor complex via the association of src kinases with accessory or coreceptor proteins. Even though members of the src family of kinases are highly related, only two src family kinases have been shown to directly bind to the ITAM, p59^{fyn}, and p56^{lyn} (Clark et al., 1992; Gauen et al., 1992; Pleiman et al., 1994). p59^{fyn} is expressed in T and B cells and associates with both the T cell and B cell antigen receptors (Samelson et al., 1990; Gassmann et al., 1992; Clark et al., 1992). p56^{lyn} is expressed in B cells and mast cells, and has been shown to bind to the B cell antigen receptor as well as to the IgE receptor (Clark et al., 1992; Jouvin et al., 1994). For the last several years, we have focused on defining what mediates the specificity of these interactions.

We previously demonstrated that the SH4 domain of p59^{fyn} confers its specific binding to TCR and BCR ITAMs (Gauen et al., 1992; Pleiman et al., 1994). In this study, we identified the critical features of the p59^{fyn} SH4 domain that are required for this binding. We tested a panel of mutated p59^{fyn} proteins with alanine substitutions for each residue in the SH4 domain. Surprisingly, we found that only the glycine residue at position two and the cysteine residue at position three were critical for binding. Although these residues were necessary for TCR binding, they are not sufficient because p56^{lck}, which cannot bind the TCR (Gauen et al., 1992), also contains a glycine at position two and a cysteine at position three. This suggested that additional residues in the SH4 domain are important for p59^{fyn} interactions with the TCR. Further mutagenesis demonstrated that two lysine residues at positions 7 and 9 of p59^{fyn}, which are not present in p56^{lck}, were also critical (Fig. 1 *A*). Although mutation of either lysine alone had little or no effect on TCR binding, mutation of both residues together completely abrogated TCR binding. Our results suggest that three features of the p59^{fyn} SH4 domain are critical for binding to the ITAM: myristoylation, palmitoylation, and basic residues in positions 7 and 9. The only other src kinase that contains all three of these features is p56^{lyn}. Since p56^{lyn} is the only other src kinase that is known to bind to the ITAM sequence, the features that we have defined are likely to constitute a specific binding motif for ITAMs. It is possible that other src family members whose SH4 domains have sites for palmitoylation and basic residues will be capable of binding to ITAMs.

It is still possible that the site of palmitoylation of p59^{fyn} and p56^{lck} is distinct and that this accounts for differential binding to the ITAM. p56^{lck} is known to be palmitoylated, but the site of palmitoylation is uncertain. It was reported that cysteine 5, cysteine 3, or most recently, both cysteines 3 and 5 are the primary sites of palmitoylation in vivo (Rodgers et al., 1994; Shenoy-Scaria et al., 1994; Yurchak and Sefton, 1995). Thus, it is conceivable that the position of the palmitate and/or possibly the number of palmitates (one versus two) precludes the ability of p56^{lck} to bind to the TCR. Other src family members are known to be palmitoylated (Shenoy-Scaria et al., 1994), however, and do not bind the ITAM. It seems more likely that the inability of p56^{lck} to bind results from the lack of the lysine residues rather than from the position of the palmitate.

It is interesting that the mutated p59^{fyn} protein lacking Cys3 was localized only to intracellular membranes. This result suggests that palmitoylation is not required for membrane binding per se, but may play a role in intracellular transport or in the plasma membrane localization of p59^{fyn}. For example, palmitoylation of p59^{fyn} may be required for exit of p59^{fyn} from intracellular membranes, or it may be required to stabilize the binding of p59^{fyn} to plasma membranes. Since the lipid composition of different cellular membranes is distinct, it is interesting to speculate that fatty acylation of initially soluble proteins might be critical for their ability to associate with a specific membrane. For example, as the cholesterol content of the membrane increases in the Golgi and plasma membrane, the lipid bilayer becomes more rigid and thickens, favoring the incorporation of longer chain hydrocarbons (reviewed in Bretscher and Munro, 1993). Another difference in membranes regulating p59^{fyn} binding might be the composition of phospholipids. The presence of basic residues or dual fatty acylation significantly enhances the binding of peptides to acidic phospholipids versus neutral phospholipids (Sigal et al., 1994; Shahinian and Silvius, 1995). Differences in the acidic phospholipid composition of plasma membranes might therefore favor the requirement for myristate (C14:0), palmitate (C16:0) and basic residues. Thus, based on physical properties, fatty acylation of p59^{fyn} could regulate its ability to associate with Golgi or plasma membranes. Indeed, it was reported that the primary cellular localization of endogenous p59^{fyn} in Jurkat cells and mitogen stimulated peripheral blood T cells was intracellular (Ley et al., 1994). This might reflect a pool of p59^{fyn} molecules that is not palmitoylated. Regulation of fatty acylation could therefore serve to dictate when p59^{fyn} is transported from internal membranes to the plasma membrane. This could be important in regulating the assembly of p59^{fyn} with the TCR or to regulate the stoichiometry of such complexes at the plasma membrane.

Given that dually acylated peptides bind tightly to membranes (Sigal et al., 1994; Shahinian and Silvius, 1995), it was surprising that the K7,9A mutant that was palmitoylated and presumably myristoylated was found to be cytosolic both by cell fractionation and by confocal microscopy. The simplest explanation is that the requirements for membrane stability of a 50–60-kD protein differ from the requirements of small peptides. More likely, we suspect that it reflects a low stoichiometry of myristoylation and palmitoylation of the K7,9 protein. Given the high levels of expression of K7A and K7,9A achieved in our ex-

periments, the level of myristate and palmitate labeling suggests that the incorporation of fatty acids was less efficient than for wild-type p59^{fyn}. Our localization studies may reflect a large portion of the molecules that are not dually modified. We also consistently noted that accumulated protein expression of the G2A, K7A, and K7,9A proteins was substantially greater than the other mutated or wild-type p59^{fyn} proteins. Myristoylation might be a rate-limiting step in the biosynthesis of these proteins.

Fatty acylation of proteins enhances their ability to bind membranes, but may have other functions. It is clear that covalent lipid modifications are implicated in the specificity of a growing number of protein–protein interactions (Linder et al., 1991; Pitcher et al., 1992; Kokame et al., 1992; Kuroda et al., 1993). This has been demonstrated in studies of subunit association of heterotrimeric G proteins. Myristoylation of purified recombinant $G_{o\alpha}$ increases its affinity for binding to βγ subunits (Linder, et al., 1991). Similarly, myristoylation and palmitoylation of p59^{fyn} may increase its affinity for binding to TCR subunits. A direct test of the role of palmitate in facilitating binding of p59^{fyn} to TCR proteins awaits the reconstitution of binding using purified components.

Intriguingly, palmitoylation is a modification that can be dynamically modulated by extracellular signals. Recently, it was demonstrated that palmitoylation of a G protein α subunit is modulated by treatment of cells with β-adrenergic receptor agonists (Degtyarev et al., 1993; Mumby et al., 1994; Wedegaertner and Bourne, 1994). If palmitoylation facilitates protein–protein interactions, cycles of acylation and deacylation could have an important regulatory role in many signaling pathways. Based on the data presented here, the modulation of p59^{fyn} palmitoylation could have an important role in enhancing or desensitizing TCR signaling by enhancing or inhibiting p59^{fyn} association with the TCR. It will be interesting to determine whether signaling by the TCR can modulate the palmitoylation state of p59^{fyn}.

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