Multiple Features of the p59\textsuperscript{fn} 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\textsuperscript{fn} 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\textsuperscript{fn} because two related src family kinases, p60\textsuperscript{src} and p56\textsuperscript{ck}, do not bind to ITAMs. In this study, we identify the residues of p59\textsuperscript{fn} that are required for binding to ITAMs. We previously demonstrated that the first 10 residues of p59\textsuperscript{fn} 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\textsuperscript{fn} correlates with its ability to bind ITAMs. Four residues (Gly2, Cys3, Lys7, and Lys9) were required for efficient binding of p59\textsuperscript{fn} to the TCR. Interestingly, the same four residues are present in p56\textsuperscript{ck}, 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\textsuperscript{fn}. Thus, the signals that direct p59\textsuperscript{fn} 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\textsuperscript{fn} and p56\textsuperscript{ck}, 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\textsuperscript{fn} is directly associated with the TCR subunits by binding to the signaling motif known as the immune-receptor tyrosine-based activation motif (ITAM)\textsuperscript{1} (Samelson et al., 1990; Gassmann et al., 1992; Gauen et al., 1992, 1994), whereas p56\textsuperscript{ck} 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\textsuperscript{ck} with CD4 and CD8 and between p59\textsuperscript{fn} and TCR subunits are mediated by these unique domains. In the case of p56\textsuperscript{ck}, 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\textsuperscript{fn}, 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.

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
SH domain that mediate its association with TCR subunits. Site-directed mutagenesis was used to generate a panel of p59
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
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
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 \( \zeta \) chains (designated as Ge and G\(_{\zeta} \), respectively) were described previously (Gauen et al., 1992). The following DNA constructs were also previously described: p59
, which encodes the hematopoietic-specific isoform of mouse p59
, fyn/myc, which encodes a myc epitope-tagged form of p59
, and srcl0/myc, which encodes a myc epitope-tagged form of p59
 in which the first 10 amino acids of chicken p60 c'src were substituted for that of p59
 (Gauen et al., 1992). The DNA constructs encoding the p59
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'CGGATCCCTAGGGCGCTGTGTGCAATGTGAA
(G2A). 5'CGAATTCTATGATGGCGCTGTGCAATGTGAA
(G2A), 5'CGAATTCTATGATGGCGCTGTGCAATGTGAA
(G2A), 5'CGAATTCTATGATGGCGCTGTGCAATGTGAA
(G2A), 5'CGAATTCTATGATGGCGCTGTGCAATGTGAA
(G2A) were detected by in vitro kinase reactions of the VSV G protein. The 3H-labeled proteins were analyzed as described (Linder et al., 1993) with minor modifications. Proteins were separated by electrophoresis. The proteins were transferred to nitrocellulose and immunoblotted using monoclonal antibodies specific for p59
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**Immunofluorescent Microscopy**

HeLa cells were transfected with the indicated DNA constructs and analyzed 4–6 h later. The cells were observed with a confocal microscope (BioRad Laboratories, Hercules, CA).

**3H-Fatty Acid Labelings**

HeLa cells were transfected with the indicated DNA constructs in triplicate. 5-6 h after transfection, the cells were fixed with 3% paraformaldehyde and permeabilized with 1% NP-40 (Sigma). The antibodies and antibodies were detected by immunoprecipitation. Myc epitope immunoblots were performed using polyclonal antiserum to p59
. Immunoblotting was performed for the binding assays on lysates from duplicate cultures of cells as previously described (Gauen et al., 1992). After the addition of 1% NP-40, 0.4% CHAPS (Pierce Chemical Co., Rockford, IL), 1% sodium deoxycholate (Sigma), 25 mM Tris, pH 6.8, 25 mM NaHPO\(_4\), 150 mM NaCl, 2.7 mM KCl) and 25 mM glucose and incubated for 20 min at room temperature with an FITC-conjugated anti-mouse secondary antibody (U.S. Biochemical Corp., Cleveland, OH). The fluorescent staining was visualized using a confocal microscope.

**3H-Fatty Acid Analysis**

Radioactive fatty acids liberated by alkaline hydrolysis of the p59
proteins were analyzed as described (Linder et al., 1993) with minor modifications. Proteins were solubilized in a polysaccharide gel slice washed with 1.5 M NaOH, and fatty acids were extracted with chloroform/methanol. After the addition of 30 mg of palmitic acid as a carrier, extracted fatty acids were analyzed by HPLC on a Beckman Ultraseph C\(_8\) reversed-phase column with acetonitrile/0.1% trifluoroacetic acid (80:20) (Beckman Instruments, Palo Alto, CA); fatty acids were also analyzed by TLC on C\(_8\) reversed-phase plates (Whatman, Clifton, NJ) with acetonitrile/acetic acid (90:10) as the mobile phase.
A

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\begin{array}{llllllllll}
\text{Residue} & A & N & D & K & E & R & L & I & N & T \\
\text{Source} & 1 & 7 & 8 & 9 & 10 & 11 & 12 & 13 & 14 & 15 \\
\text{Lyn} & G & M & C & V & C & K & K & R & K & E \\
\text{Ick} & M & C & G & C & N & K & S & K & R & N \\
\text{Ig} & C & G & C & K & K & T & C & T & K & E \\
\text{Hck} & M & C & M & W & K & S & F & L & Q & D \\
\text{Btk} & M & G & L & S & S & K & K & O & V & Q \\
\text{Src} & M & G & S & S & K & S & K & P & K & D \\
\end{array}
\]


B

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\begin{array}{llllllllll}
\text{Substitution} & A & N & D & K & E & R & L & I & N & T \\
\text{Source} & 1 & 7 & 8 & 9 & 10 & 11 & 12 & 13 & 14 & 15 \\
\text{Lyn} & G & M & C & V & C & K & K & R & K & E \\
\text{Ick} & M & C & G & C & N & K & S & K & R & N \\
\text{Ig} & C & G & C & K & K & T & C & T & K & E \\
\text{Hck} & M & C & M & W & K & S & F & L & Q & D \\
\text{Btk} & M & G & L & S & S & K & K & O & V & Q \\
\text{Src} & M & G & S & S & K & S & K & P & K & D \\
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\text{Figure 1.} Analysis of the amino-terminal residues of p59fn. (A) Comparison of the amino-terminal residues of src family kinases. (B) Diagram of p59fn proteins containing single amino acid substitutions. The amino-terminal residues of p59fn are indicated. The position of each amino acid is designated numerically from the initiator methionine. A series of nine p59fn proteins was generated, each of which contains an alanine substitution at the indicated position.

Results

Determination of Critical Residues of p59fn for Binding to TCR Proteins

We previously demonstrated that p59fy associates with multiple TCR subunits. All these subunits contain a signaling motif known as the ITAM, and we showed that p59fy interacts directly with the ITAM (Gauen et al., 1994). This interaction is specific for p59fy because two highly related src family kinases, p56lck and p60fyn, did not bind to the chimeric TCR proteins. Mapping studies revealed that a small region of p59fy 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. 1A). We were therefore interested in determining the unique features of the p59fy 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 p59fy proteins containing single alanine substitutions were generated (depicted in Fig. 1B). 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 p59fy. All of the mutated p59fn 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, Ge, which contains the extracellular domain of the VSV G fused to the cytoplasmic domain of CD3e. Association of p59fn 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 p59fn and Ge result in phosphorylated proteins migrating with apparent molecular mobilities of 75 and 65 kD, representing phosphorylated Ge and p59fn, respectively (Gauen et al., 1992, and Fig. 2A, lane 1). We also reported previously that this binding is 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 Ge (Gauen et al., 1992, 1994). Little or no kinase activity was detected in the VSV G immunoprecipitates from cells that coexpress Ge with the G2A or C3A proteins (Fig. 2A, lanes 2 and 3). The remainder of the p59fn proteins retained the ability to bind Ge (Fig. 2A, lanes 4–10). Identical results were obtained when a fusion protein containing the cytoplasmic domain of another TCR subunit, Gz (Gauen et al., 1992), was used (data not shown). Immunoblotting of whole-cell lysates with antibodies to VSV and p59fn demonstrated that all of the proteins were highly expressed (Fig. 2B and C, lanes 1–10), confirming that the inability of G2A and C3A to associate with Ge was not caused by lower levels of expression. We were concerned that the detection of K7A kinase activity coprecipitating with Ge was caused by higher levels of K7A expression. Even when K7A was ex-

\text{Figure 2.} Association of mutated p59fn proteins with Ge. (A) In vitro kinase reactions of VSV G immunoprecipitations. HeLa cells were cotransfected with Ge and the indicated p59fn 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 32P-labeled proteins were electrophoretically separated on SDS-polyacrylamide gels and detected by autoradiography. (B and C) VSV and p59fn 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 p59fn (C, lanes 1–14), 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 Ge (75 kD) and p59fn (65 kD) proteins are indicated.
pressed at levels lower than p59fyn, however, coprecipitating kinase activity was observed (data not shown). These data confirmed that K7A can bind Ge as efficiently as p59fyn. 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 p59fyn Binding to the ITAM**

Our data pointed to two residues of the p59fyn SH4 domain, Gly2 and Cys3, as the critical residues for binding. p56lck, 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 p59fyn SH4 domain are lysines at positions 7 and 9 and a cysteine residue at position 3. In contrast, the p56lck SH4 domain does not contain any lysine residues and the SH4 domain of p60src (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 p59fyn SH4 domain to bind to TCR subunits. To test this hypothesis, we substituted a cysteine residue for Ser3 in the srcl0/fyn protein, where the SH4 domain of p60src had been substituted for that of p59fyn. The srcl0/Ser3 protein was able to associate with Ge as efficiently as p59fyn, whereas srcl0/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 p59fyn (K7,9A). Although K7,9A was expressed at comparable levels to p59fyn (Fig. 2 C, lanes 13 and 14), the loss of both lysines greatly reduced binding to Ge (Fig. 2 A, lanes 13 and 14). Therefore, the combination of Cys3 with Lys7 and Lys9 in the SH4 domain of p59fyn is responsible for its unique ability to direct binding to TCR subunits. These data suggest that the failure of p56lck to bind TCR proteins is caused by the lack of two lysine residues in its SH4 domain.

**Fatty Acylation of p59fyn Proteins**

All src family kinases are N-myristoylated at Gly2, and with the exception of p556 and p60src, may be palmitoylated at neighboring cysteine residues (Paige et al., 1993; Koegl et al., 1994; Resh, 1994). The observation that residues of p59fyn 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 p59fyn proteins. To confirm that the myc epitope–tagged p59fyn expressed in our system was palmitoylated, HeLa cells expressing this protein were incubated with radioactive palmitate. Radioactivity derived from [3H]palmitate was incorporated into epitope-tagged p59fyn, but not into epitope-tagged srcl0/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 p59fyn expressed in our system is palmitoylated.

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 p59fyn (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 srcl0/fyn (Fig. 3 B, closed circles). Thus, noncovalently associated radioactivity was not carried through the immunoprecipitation and extraction pro-

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**Figure 3.** Incorporation of [3H]palmitate into p59fyn proteins in HeLa cells. (A) HeLa cells transfected with a cDNA encoding myc epitope–tagged p59fyn (lanes 1 and 3) or myc epitope–tagged srcl0/fyn (lanes 2 and 4) were incubated with [3H]palmitate. p59fyn immunoprecipitates were prepared from cell lysates and separated by SDS-PAGE. [3H]Palmitate incorporation was detected by fluorography (lanes 1 and 2). A portion of each lysate was analyzed by immunoblotting with antisera to p59fyn (lanes 3 and 4), as described in Fig. 2 C. (B) Reversed-phase chromatography of fatty acids hydrolyzed from radiolabeled myc epitope–tagged p59fyn and srcl0/fyn. Immunoprecipitates of p59fyn proteins from transfected HeLa cells were resolved by SDS-PAGE. Gel slices containing p59fyn or srcl0/fyn were excised and subjected to base hydrolysis. Extracts of the hydrolysates were chromatographed over a C18 reversed-phase column, and radioactive fatty acids were detected by scintillation counting. Elution profiles of the p59fyn extracts (open squares) and the srcl0/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.
Figure 4. 3H-fatty acid labeling of p59<sup>fyn</sup> proteins. (A) [3H]Myristate and [3H]palmitate labeling of myc epitope-tagged p59<sup>fyn</sup> proteins. HeLa cells were transfected with the indicated DNA constructs and incubated with [3H]myristate (lanes 1-4) or [3H]palmitate (lanes 5-12). The incorporated radioactivity was detected in p59<sup>fyn</sup> immunoprecipitates by SDS-PAGE and fluorography. (B) Immunoblotting of p59<sup>fyn</sup> immunoprecipitates from A. A portion of each immunoprecipitate from the [3H]myristate-labeled cells (lanes 1-4) or from the [3H]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<sup>fyn</sup> 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.

To determine whether there was a correlation between fatty acylation and the ability to bind TCR subunits, the mutated p59<sup>fyn</sup> proteins were tested for metabolic incorporation of [3H]myristate and [3H]palmitate (Fig. 4 A, lanes 1-4 and 5-12, respectively). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. As expected, p59<sup>595</sup> and C3A were labeled after [3H]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 [3H]myristate, suggesting that unlike for p60<sup>src</sup> (Kaplan et al., 1988), Lys7 is not absolutely required for myristoylation of p59<sup>fyn</sup> (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<sup>fyn</sup>. Because the amount of [3H]myristate incorporated into K7A was equal to or less than wild-type p59<sup>fyn</sup> (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<sup>fyn</sup> to be myristoylated.

Metabolic incorporation of [3H]palmitate revealed efficient labeling of the p59<sup>fyn</sup>, 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<sup>fyn</sup> 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 [3H]palmitate (data not shown). Since all the p59<sup>fyn</sup> 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<sup>fyn</sup> Proteins

To determine if the ability of the mutated p59<sup>fyn</sup> proteins to bind TCR proteins correlated with their ability to associate with membranes, the membrane stability of p59<sup>fyn</sup> and the p59<sup>fyn</sup> 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<sup>fyn</sup> 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<sup>fyn</sup> 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<sup>fyn</sup> proteins did not absolutely reflect their ability to bind TCR subunits.

It was possible that the redistribution 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<sup>fn</sup> 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<sup>fn</sup> 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<sup>fn</sup>, and p56<sup>lk</sup> (Clark et al., 1992; Gauen et al., 1992; Pleiman et al., 1994). p59<sup>fn</sup> 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<sup>lk</sup> 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<sup>fn</sup> 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<sup>fn</sup> SH4 domain that are required for this binding. We tested a panel of mutated p59<sup>fn</sup> 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<sup>lk</sup>, 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<sup>fn</sup> interactions with the TCR. Further mutagenesis demonstrated that two lysine residues at positions 7 and 9 of p59<sup>fn</sup>, which are not present in p56<sup>lk</sup>, 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<sup>fn</sup> 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<sup>lk</sup>. Since p56<sup>lk</sup> 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<sup>fn</sup> and p56<sup>lk</sup> is distinct and that this accounts for differential binding to the ITAM. p56<sup>lk</sup> 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<sup>lk</sup> 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<sup>lk</sup> 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<sup>fn</sup> 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<sup>fn</sup>. For example, palmitoylation of p59<sup>fn</sup> may be required for exit of p59<sup>fn</sup> from intracellular membranes, or it may be required to stabilize the binding of p59<sup>fn</sup> 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<sup>fn</sup> 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<sup>fn</sup> could regulate its ability to associate with Golgi or plasma membranes. Indeed, it was reported that the primary cellular localization of endogenous p59<sup>fn</sup> in Jurkat cells and mitogen stimulated peripheral blood T cells was intracellular (Ley et al., 1994). This might reflect a pool of p59<sup>fn</sup> molecules that is not palmitoylated. Regulation of fatty acylation could therefore serve to dictate when p59<sup>fn</sup> is transported from internal membranes to the plasma membrane. This could be important in regulating the assembly of p59<sup>fn</sup> 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<sub>fyn</sub>. 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<sub>fyn</sub> 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 rate-limiting step in the biosynthesis of these proteins.

or wild-type p59<sub>fyn</sub> proteins. Myristoylation might be a covalent lipid modifications are implicated in the specific-function of the G2A, K7A, and K7,9A proteins was substantially greater than the other mutated protein expression of the G2A, K7A, and K7,9A proteins. Myristoylation might be a rate-limiting step in the biosynthesis of these proteins.

Similarly, myristoylation and palmitoylation of p59<sub>fyn</sub> may increase its affinity for binding to TCR subunits. A direct test of the role of palmitate in facilitating binding of p59<sub>fyn</sub> 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<sub>fyn</sub> palmitoylation could have an important role in enhancing or desensitizing TCR signaling by enhancing or inhibiting p59<sub>fyn</sub> association with the TCR. It will be interesting to determine whether signaling by the TCR can modulate the palmitoylation state of p59<sub>fyn</sub>.

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