

Cysteine³ of Src Family Protein Tyrosine Kinases Determines Palmitoylation and Localization in Caveolae

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Abstract. Recent work has demonstrated that p56^{lck}, a member of the Src family of protein tyrosine kinases (PTKs), is modified by palmitoylation of a cysteine residue(s) within the first 10 amino acids of the protein (in addition to amino-terminal myristoylation that is a common modification of the Src family of PTKs). This is now extended to three other members of this family by showing incorporation of [³H]palmitate into p59^{lyn}, p55^{lck}, and p56^{hck}, but not into p60^{src}. The [³H]palmitate was released by treatment with neutral hydroxylamine, indicating a thioester linkage to the protein. Individual replacement of the two cysteine residues within the first 10 amino acids of p59^{lyn} and p56^{lck} with serine indicated that Cys³ was the major determinant of palmitoylation, as well as association of

the PTK with glycosyl-phosphatidylinositol-anchored proteins. Introduction of Cys³ into p60^{src} led to its palmitoylation. p59^{lyn} but not p60^{src} partitioned into Triton-insoluble complexes that contain caveolae, microinvaginations of the plasma membrane. Mapping of the requirement for partitioning into caveolae demonstrated that the amino-terminal sequence Met-Gly-Cys is both necessary and sufficient within the context of a Src family PTK to confer localization into caveolae. Palmitoylation of this motif in p59^{lyn} also modestly increased its overall avidity for membranes. These results highlight the role of the amino-terminal motif Met-Gly-Cys in determining the structure and properties of members of the Src family of PTKs.

THE Src family of protein tyrosine kinases (PTKs)¹ is comprised of nine members, whose prototype p60^{src} was first discovered as the transforming gene of Rous sarcoma virus and later found to have as its normal cellular counterpart the protooncogene p60^{c-src} (3). Many of the members of the Src family are involved in signal transduction and cell activation, with p56^{lck} and p59^{lyn} being well analyzed examples. Several lines of evidence have implicated p56^{lck} and p59^{lyn} in lymphocyte activation through the T cell antigen receptor (17). These results correlate with the biochemical findings of a direct association between p59^{lyn} and the CD3/ζ chain complex of the T cell receptor (32, 42) and between p56^{lck} and the coreceptors CD4 and CD8 (31, 43).

Activation of T cells also occurs after crosslinking of glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins (21, 28). Analysis of the mechanism of lymphocyte activation in this case has led to new insights into the features of GPI-anchored proteins and their role in the cell. The GPI

anchor does not traverse the lipid bilayer of the plasma membrane, so it cannot directly transmit a signal into the cell. The observation that GPI-anchored proteins are associated with the Src family protein tyrosine kinases p56^{lck} and p59^{lyn} (40, 37, 41) indicates a possible pathway for signaling through the GPI-anchored proteins. The GPI-anchored proteins are relatively insoluble in Triton X-100 detergent, remaining mostly as large complexes or lipid subdomains that are enriched in glycosphingolipids and GPI-anchored proteins (5, 7). Recent ultrastructural and biochemical data provide strong evidence that these Triton-insoluble complexes contain plasma membrane microinvaginations called caveolae (1). Specifically, GPI-anchored proteins are concentrated in both Triton-insoluble complexes (5, 7) and in caveolae (30, 46). Purification of these complexes yields structures that are enriched in caveolin, a marker protein for caveolae (29), and that have the ultrastructural appearance of caveolae (35).

We have recently demonstrated that the complex between GPI-anchored proteins such as decay-accelerating factor (DAF, CD55) and p56^{lck} or p59^{lyn} depends on an amino-terminal cysteine motif of these PTKs, both of which have two cysteine residues in the first 10 amino acids (38). Direct radiolabeling of p56^{lck} with [³H]palmitate demonstrated that this was based on palmitoylation of an amino-terminal cysteine residue(s) (38). Myristoylation of the amino-terminal

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1. *Abbreviations used in this paper:* DAF, decay-accelerating factor; GPI, glycosyl-phosphatidylinositol; MDCK, Madin-Darby canine kidney (cells); PTK, protein tyrosine kinases.

glycine residue, a common modification present in all members of the Src family of PTKs, was also necessary for association of DAF and the PTK (38).

The simplest model to encompass these findings is that palmitoylation and myristoylation of a Src family PTK leads to localization into caveolae, bringing the GPI-anchored protein and the signaling molecule together to serve in an activation pathway. In the present study, we directly examined the localization of the Src family PTKs, and we demonstrated that Cys³ (present in p56^{lck} and p59^{lyn} but not p60^{src}) determines palmitoylation of the PTK and leads to its inclusion in caveolae. The amino-terminal sequence Met-Gly-Cys thus defines a novel motif for dual acylation of a protein with resultant structural and functional consequences.

Materials and Methods

Cells and Antibodies

HeLa and Madin-Darby canine kidney (MDCK) cells were maintained in α -MEM, and murine thymoma EL-4 cells in RPMI 1640 (Gibco Laboratories, Grand Island, NY), each fortified with 10% FCS, 2 mM glutamine, 50 μ g/ml streptomycin, and 50 U/ml penicillin in a 5% CO₂/95% air atmosphere at 37°C. The mouse mAb to human DAF, 1H4 (8), was a gift from W. Rosse (Duke University, Durham, NC). Mouse mAbs to p60^{src} and caveolin were obtained from Oncogene Science (Uniondale, NY) and Transduction Laboratories (Lexington, KY), respectively. Rabbit polyclonal antiserum to p59^{lyn} and p56^{lck} used for immunoprecipitation was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity-purified rabbit IgG against p59^{lyn} and p56^{lck} used for Western blotting and immunofluorescence studies, and against p56^{lck} used for immunoprecipitations, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antiserum to p55^{fer} was raised against a recombinant protein containing amino acids 1–87 of human p55^{fer}, and affinity purified before use (Link, D.C., manuscript submitted for publication).

DNA Constructs and Mutagenesis

Amino acid substitutions in p60^{src}, p59^{lyn}, and p56^{lck} were achieved by polymerase chain reaction using an oligonucleotide encoding the desired mutation and plasmids encoding the wild-type forms of chicken p60^{src}, murine p59^{lyn}, and murine p56^{lck}, respectively (38). Accuracy of the mutations was confirmed by DNA sequence analysis (33). Human fgr cDNA (34) was provided by Keith Robbins (National Institute of Dental Research, Bethesda, MD). The human hck cDNA was cloned from a human monocyte cDNA library (Link, D. C., unpublished data). To improve translational efficiency, the sequences immediately upstream of the initiation ATG codon were modified to conform to the Kozak consensus, resulting in the exclusive expression of p56^{lck} by this construct.

Transfections

HeLa cells in 10-cm petri dishes were infected with recombinant vaccinia virus expressing the T7 RNA polymerase (13) for 30 min in serum-free media. 10 μ g of the DNA of interest under control of the T7 promoter was then added to the cells together with 30 μ g lipofectamine (GIBCO BRL, Gaithersburg, MD). Amounts of virus, DNA and lipofectamine were reduced for smaller scale transfections. Cells were analyzed 16 h after infection.

HeLa transfectants stably expressing p59^{lyn} and EL-4 transfectants stably expressing human DAF or a chimeric transmembrane version of DAF have been described previously (37). MDCK cells stably expressing DAF were produced by cotransfection of 15 μ g human DAF cDNA (8) and 1 μ g neomycin resistance gene, both in the spleen focus forming virus expression vector (8), together with 100 μ g lipofectin. Cells were treated with 0.75 μ g/ml G418, and individual colonies were selected and expanded.

Immunoprecipitation and Immune Complex Kinase Assay

Transfected HeLa cells were washed twice in cold phosphate-buffered saline

and lysed at a density of 5–10 \times 10⁶ cells/ml in a buffer containing 0.5% Triton X-100, 50 mM Tris-HCl, (pH 7.6), 300 mM NaCl, 1 mM sodium orthovanadate, 5 mM EDTA, 10 mM iodoacetamide, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin. Lysates were precleared and the protein of interest was immunoprecipitated as described previously (22) using the immunoabsorbent Pansorbin (Calbiochem-Behring Corp., San Diego, CA). Immunoprecipitates were washed three times in lysis buffer without EDTA and resuspended in 50 μ l of buffer containing 25 mM Hepes (pH 7.4), 3 mM MnCl₂, 0.1 mM sodium orthovanadate, and 10 μ Ci [γ -³²P] ATP (New England Nuclear, Boston, MA). Kinase reactions proceeded for 15 min at room temperature and were terminated with EDTA. Immunoprecipitates were then washed in 0.5% Triton X-100 lysis buffer, and protein was eluted in 1X reducing Laemmli buffer and analyzed on sodium dodecyl sulfate–9% polyacrylamide gels.

Biosynthetic Labeling

4 h after viral infection, HeLa cells were labeled with 0.2–0.5 mCi/ml [9,10-³H]palmitate or 0.1 mCi/ml [9,10-³H]myristate (New England Nuclear) and incubated for another 12 h. Cells were subsequently lysed, immunoprecipitated as described, and analyzed by SDS-PAGE and fluorography. In some cases, before fluorography, replicate gel slices were soaked overnight in either 1 M hydroxylamine (pH 7.5) or 1 M Tris-HCl (pH 7.5).

Analysis of Thioester-linked Fatty Acids

Cells were labeled with [³H]palmitate and cell lysates were immunoprecipitated as described above. The proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The p59^{lyn} band was localized by Western blotting, excised, and subjected to alkaline hydrolysis in 1 ml of 1.5 N NaOH for 3 h at 30°C. The extract was acidified with HCl (pH 1–2), extracted twice with toluene, and dried completely under N₂. The material was redissolved in ethanol and analyzed on a C-18 reverse phase thin-layer chromatography plate developed with glacial acetic acid/acetone/nitrile (1:1). Authentic standards of [³H]palmitate and [³H]myristate were run on the plate and located by autoradiography. The corresponding positions from the sample lane were scraped and counted in a liquid scintillation counter.

Detection of Detergent-insoluble Lipid Subdomains (Caveolae)

Caveolae were isolated by a modification of a Sepharose 4B column chromatography assay described by Cinek and Horejsi (7). Briefly, cells were lysed at a density of 10⁷/ml in the immunoprecipitation lysis solution buffered to pH 6.5 with 50 mM Pipes. 300 μ l of the lysate was applied to a preequilibrated 1 \times 5-cm Sepharose 4B column maintained at 4°C, and the eluate was discarded. Seven 0.5-ml fractions were collected after consecutive applications of 0.5 ml of lysis buffer. Fractions were analyzed by SDS-PAGE followed by Western blotting. The column was calibrated using both intact and lysed erythrocytes. Intact erythrocytes eluted in fraction 2, therefore corresponding to the column void volume, while the included volume was marked by hemoglobin from lysed erythrocytes that eluted primarily in fractions 4 and 5.

Western Blotting

Proteins were transferred to 0.45- μ m nitrocellulose paper (Costar Corp., Cambridge, MA) and blocked as described (38). After a 30-min incubation with primary antibody diluted in blocking solution, the blot was incubated for an additional 30 min with appropriate secondary IgG-horse radish peroxidase conjugate. The membrane was washed three times for 5–10 min each in Tris-buffered saline + 0.05% Tween 20, and developed with ECL chemiluminescent substrate (Amersham Corp., Arlington Heights, IL).

Membrane Preparations

Cells were washed twice in phosphate-buffered saline and then suspended to a concentration of 5–10 \times 10⁶ cells/ml in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. After 15 min of swelling, cells were disrupted by 30–40 passes of a Dounce homogenizer. The homogenate was adjusted to 0.25 M sucrose and centrifuged at 1,000 g for 10 min to clear any remaining intact cells. The resulting supernatant was centrifuged for 60 min at 150,000 g. The membrane pellet was resuspended directly in 1X Laemmli sample buffer, and the supernatant was added to an appropriate volume of 3X

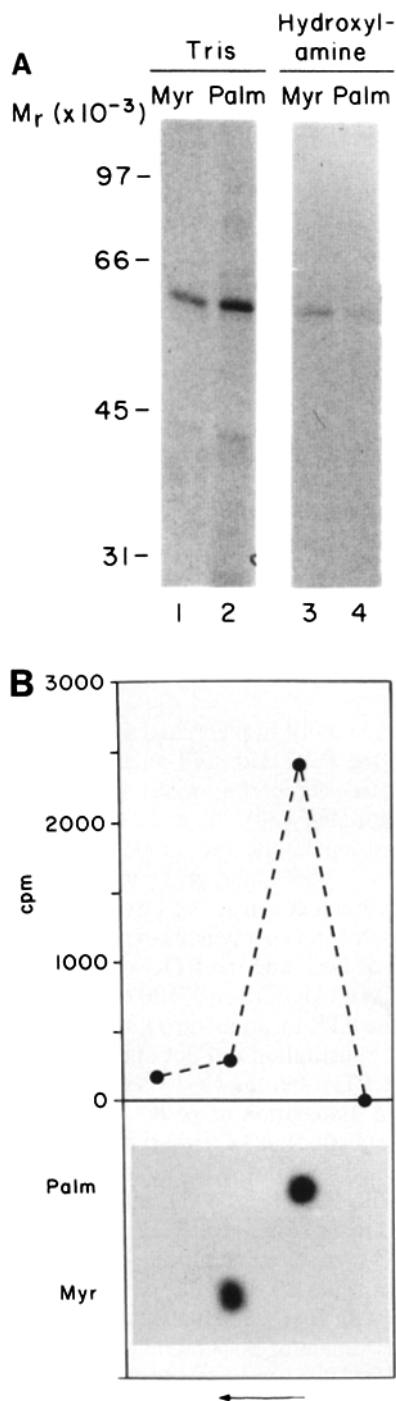


Figure 1. Biosynthetic labeling of p59^{fyn} with [³H]palmitate and [³H]myristate. HeLa cells were transiently transfected with p59^{fyn} and biosynthetically labeled with [³H]palmitate or [³H]myristate. p59^{fyn} immunoprecipitates of these radiolabeled cell lysates were analyzed by SDS-PAGE and fluorography. (A) HeLa cells expressing p59^{fyn} were labeled with [³H]myristate (lanes 1 and 3) or [³H]palmitate (lanes 2 and 4), and duplicate samples of p59^{fyn} immunoprecipitates were separated by SDS-PAGE, following which the lanes were treated overnight with either 1 M Tris-HCl, pH 7.5 (lanes 1 and 2), or 1 M hydroxylamine, pH 7.5 (lanes 3 and 4), followed by fluorography. (B) The p59^{fyn} immunoprecipitate of [³H]palmitate-labeled cells was separated by SDS-PAGE, Western blotted onto PVDF membrane, and subjected to alkaline hydrolysis. The released material was analyzed by C-18 reverse-phase thin-layer chromatography along with [³H]palmitate and [³H]myristate

Laemmli sample buffer. An equal percentage of the soluble and membrane samples were then analyzed by SDS-PAGE and Western blotting.

Immunofluorescence

This procedure was adapted from a previously described protocol (36). Briefly, HeLa cells were plated onto glass coverslips, infected as described with recombinant vaccinia virus, transfected with cDNA, and incubated for 14–16 h. Cells were fixed in methanol at -20°C for 8 min, incubated with murine anti-DAF (1H4) and rabbit anti-p59^{fyn} antibodies, followed by anti-mouse IgG-fluorescein and anti-rabbit IgG-rhodamine secondary antibodies (Sigma Immunochemicals, St. Louis, MO). Identical fields were photographed on a microscope (Optiphot; Nikon Inc., Melville, NY) using wavelengths and filters sensitive to each conjugated fluorophore.

Results

Palmitoylation of p59^{fyn}

Using a HeLa cell transient transfection system, we have recently shown metabolic labeling of p56^{lck} with [³H]palmitate, and we demonstrated that cysteine residues present at positions 3 and/or 5 were necessary for the palmitoylation (38). This amino-terminal cysteine motif is also present in p59^{fyn} (at positions 3 and 6), so to extend our findings to an additional member of the Src family of PTKs, we directly investigated palmitoylation of p59^{fyn}. HeLa cells transfected with p59^{fyn} were metabolically labeled with [³H]myristate or [³H]palmitate, and p59^{fyn} incorporated both fatty acids (Fig. 1 A). The intensities of the radiolabeled p59^{fyn} bands cannot be quantitatively compared because the amount of radiolabel, its specific activity, the endogenous pool size, and cellular processing all differed between the [³H]palmitate and [³H]myristate radiolabels. We used the different chemical linkages of myristate and palmitate to confirm the correct labeling because fatty acids may interconvert during metabolic labeling. Myristoylation occurs on an amino-terminal glycine residue through an amide linkage, and palmitoylation generally occurs on a cysteine residue through a thioester linkage. The thioester but not the amide linkage is sensitive to hydroxylamine at neutral pH, so duplicate radiolabeled p59^{fyn} immunoprecipitates were separated by SDS-PAGE and treated with either hydroxylamine or Tris, both at pH 7.5, followed by fluorography. The [³H]palmitate label was almost completely removed by hydroxylamine treatment, but the [³H]myristate was resistant to hydroxylamine treatment (Fig. 1 A), thus demonstrating the distinct identities of the two labels and the thioester linkage of the palmitate. Next, the fatty acid was removed from the [³H]palmitate-labeled p59^{fyn} (after transfer to polyvinylidenedifluoride membrane) by alkaline hydrolysis and was analyzed by thin-layer chromatography on a C-18 reverse phase plate. This confirmed the released fatty acid as palmitate (Fig. 1 B). Thus, we conclude that p59^{fyn} is modified by thioester-linked palmitate.

Cys³ Residue of p59^{fyn} and p56^{lck} is the Major Determinant of Palmitoylation of the PTKs

We had previously shown that palmitoylation of p56^{lck} re-

standards, as described in Materials and Methods. The counts per minute scraped from each area of the p59^{fyn} lane are shown in the graph over the autoradiograph of the corresponding positions on the two standard lanes. The arrow shows the direction of chromatography.

quires the amino-terminal cysteine motif, as mutation of these two cysteine residues to serine abolished palmitoylation (38). To extend this finding to p59^{fn}, we constructed a p59^{fn} mutant with the substitutions Cys^{3,6}→Ser, and expressed that mutant along with wild-type p59^{fn} and a p59^{fn}(Gly²→Ala) mutant in HeLa cells (the Gly² residue is an absolute requirement for myristoylation of a protein, so this latter mutant is not myristoylated). The Cys^{3,6}→Ser substitution abolished [³H]palmitate incorporation into p59^{fn} (Fig. 2), but [³H]myristate was still incorporated into this mutant (see Fig. 10 in reference 38). Hence, the amino-terminal cysteine motif is critical to palmitoylation of p59^{fn}. Furthermore, the p59^{fn}(Gly²→Ala) mutant did not incorporate either fatty acid radiolabel (Fig. 2; see Fig. 10 in reference 38), indicating that myristoylation is required for palmitoylation of p59^{fn}.

To determine if one or both of the amino-terminal cysteine residues are critical for palmitoylation of p59^{fn}, we constructed mutants that had individual substitutions of serine for each of these cysteine residues. These two mutants and wild-type p59^{fn} were expressed in HeLa cells and labeled with [³H]palmitate. The Cys³→Ser substitution completely

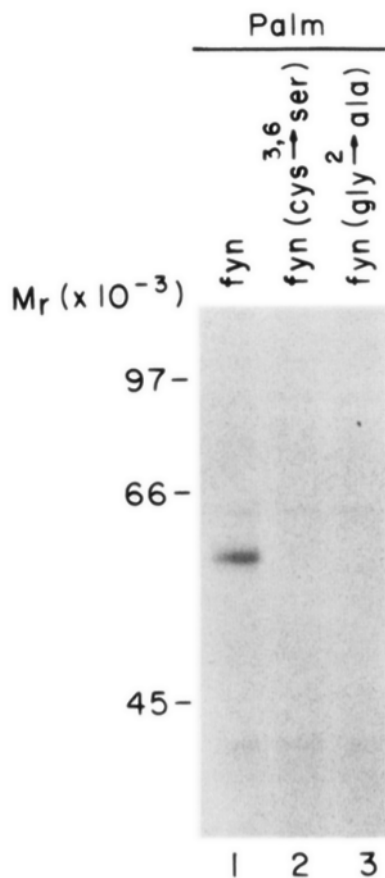


Figure 2. Palmitoylation of p59^{fn} depends on amino-terminal cysteine motif and myristoylation of p59^{fn}. HeLa cells expressing p59^{fn} (lane 1), p59^{fn}(Cys^{3,6}→Ser) (lane 2), or p59^{fn}(Gly²→Ala) (lane 3) were biosynthetically labeled with [³H]palmitate, followed by immunoprecipitation of p59^{fn} from cell lysates, separation by SDS-PAGE, and fluorography. Western blots of cell lysates with anti-p59^{fn} demonstrated approximately equal p59^{fn} expression in each cell (data not shown).

abolished incorporation of [³H]palmitate into p59^{fn}, whereas the Cys⁶→Ser substitution had no effect on [³H]palmitate incorporation (Fig. 3 A). Thus, Cys³ of p59^{fn} is critical for palmitoylation of the protein, most likely serving as the site of thioester linkage of palmitate. Since removal of both amino-terminal cysteine residues of p56^{lck} abolishes palmitoylation (38), we next analyzed the influence of each of the two amino-terminal cysteine residues separately. The Cys³→Ser substitution caused a marked (but not complete) decrease in [³H]palmitate incorporation into p56^{lck}, whereas the Cys⁵→Ser substitution had no significant effect on [³H]palmitate incorporation (data not shown). Finally, to ascertain if a cysteine at position 3 is sufficient for palmitoylation of a Src family PTK, we biosynthetically labeled HeLa cells expressing p60^{src} (Ser³→Cys) with [³H]palmitate. The wild-type p60^{src} did not incorporate [³H]palmitate, but introduction of a Cys³ residue led to labeling of p60^{src} with [³H]palmitate (Fig. 3 B). We conclude that within the context of a Src family PTK, the presence of a cysteine residue at position 3 is the critical determinant of palmitoylation.

Cys³ Residue of Src Family PTKs is the Major Determinant of Association of PTK with GPI-anchored Proteins

The amino-terminal cysteine motif in p59^{fn} and p56^{lck} is required for association of the PTK with GPI-anchored proteins such as DAF, and transfer of this motif into p60^{src} leads to its association with DAF (38). To study the role of the two cysteine residues separately, the single cysteine-serine substitutions in p59^{fn}, p56^{lck}, and p60^{src} were tested for association with DAF. Each construct was expressed in HeLa cells, which have high-level endogenous expression of DAF, and the association of DAF and the PTK was assayed by immunoprecipitating DAF from Triton X-100 cell lysates followed by detection of the PTK by an *in vitro* kinase assay. For p59^{fn}, the Cys⁶→Ser substitution did not affect the association of DAF with the PTK, but the Cys³→Ser substitution completely abolished association of p59^{fn} with DAF (Fig. 4 A). For p56^{lck}, substitution of either of the two amino-terminal cysteine residues reduced but did not abolish the interaction of p56^{lck} with DAF (Fig. 4 B), although the Cys³→Ser substitution led to a greater reduction in association with DAF than the Cys⁵→Ser substitution. For p60^{src}, introduction of a cysteine residue at position 3, but not at position 6, led to association with DAF (data not shown). Overall, Cys³ is the major determinant of association of the PTK with DAF, although in p56^{lck}, this can be supported by Cys⁵ in the absence of Cys³.

Cys³ of p59^{fn} is Necessary for Partitioning into Caveolae

We next investigated whether the presence of Cys³ in Src family PTKs can affect the subcellular localization of the protein. The data above demonstrate that Cys³ of the Src family PTKs determines palmitoylation of the protein, as well as its ability to complex with GPI-anchored proteins. Since the GPI-anchored proteins are localized to caveolae (30, 46), we reasoned that Cys³ of the Src family PTKs might determine the localization of the PTKs into caveolae. Recent biochemical and ultrastructural data (12, 35) support the idea that Triton-insoluble complexes (5), which can be

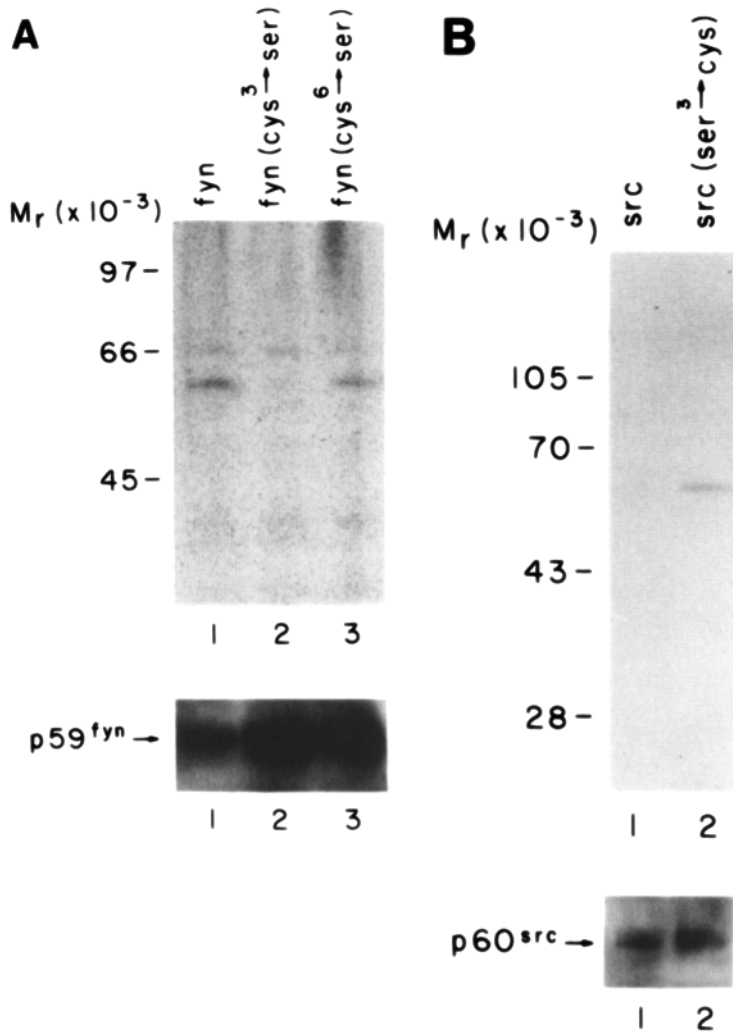


Figure 3. Cys³ residue in Src family PTK leads to palmitoylation. (A) HeLa cells expressing p59^{fyn} (lane 1), p59^{fyn} (Cys³→Ser) (lane 2), or p59^{fyn} (Cys⁶→Ser) (lane 3) were labeled and analyzed as in Fig. 2. Below the fluorograph is a Western blot of cell lysates with anti-p59^{fyn}. (B) HeLa cells expressing wild-type p60^{src} (lane 1) or p60^{src} (Ser³→Cys) (lane 2) were labeled with [³H]palmitate and analyzed as above (immunoprecipitation and Western blot with anti-p60^{src}).

isolated in the void volume of a Sepharose-4B column (7), represent a highly caveolae-enriched preparation. To provide additional evidence that the void volume fraction of the Sepharose-4B column contains caveolae, we used caveolin, a marker protein of caveolae (29). In MDCK cells transfected with human DAF, caveolin was almost entirely recovered in the void volume of the column, and DAF was found both in the void volume as well as in the included volume (Fig. 5). The percentage of the DAF molecules that are in the void volume (caveolar fraction) is consistent with results of immunoelectron microscopy, which found that for the GPI-anchored protein Thy-1, only 24% of the immunogold particle clusters were in caveolae (46). Using this biochemical assay system, the partitioning of the GPI-anchored proteins into caveolae can be demonstrated to be a property of the GPI anchor itself (Fig. 6). Wild-type human DAF expressed in murine EL-4 cells was partially contained in the Sepharose-4B column void volume (fraction 2), while a chimeric transmembrane version of DAF eluted exclusively with the included volume (fractions 4–6). Thus, the lipid modification determines the localization of DAF to caveolae, and we used this same analytical approach to determine if the palmitoylation of p59^{fyn} affects its subcellular localization.

Analysis of wild-type p59^{fyn} and p60^{src} expressed in HeLa

cells demonstrates that ~20% of p59^{fyn}, but none of p60^{src}, was recovered in the Sepharose 4B column void volume (Fig. 7). Replacement of the first 10 amino acids of p60^{src} with amino acids 1–10 of p59^{fyn} conferred on this chimeric protein (42) the ability to partition into caveolae (Fig. 8). Conversely, substitution of amino acids 1–10 of p59^{fyn} with the first 10 amino acids of p60^{src} resulted in a chimeric protein (42) that was not contained in caveolae (Fig. 8). We next examined the role of cysteine residues at positions 3 and 6 for partitioning of the PTK into caveolae. Despite similar expression, p59^{fyn}(Cys^{3,6}→Ser) did not elute in the void volume, while p60^{src}(Ser^{3,6}→Cys) did (Fig. 8). When examined individually (Fig. 9), substitution of Ser for Cys at position 3, but not at position 6, of p59^{fyn} abrogated inclusion in caveolae, while substitution of Cys for Ser at position 3, but not at position 6, of p60^{src} induced partitioning into caveolae. Cumulative evidence thus indicates that a cysteine residue at position 3 of an Src family PTK, which we have shown to be necessary for palmitoylation of the PTK, mediates the inclusion of the PTK in caveolae.

Colocalization of DAF and p59^{fyn} in Cells by Immunofluorescence

We have studied the inclusion of both DAF (Fig. 6) and

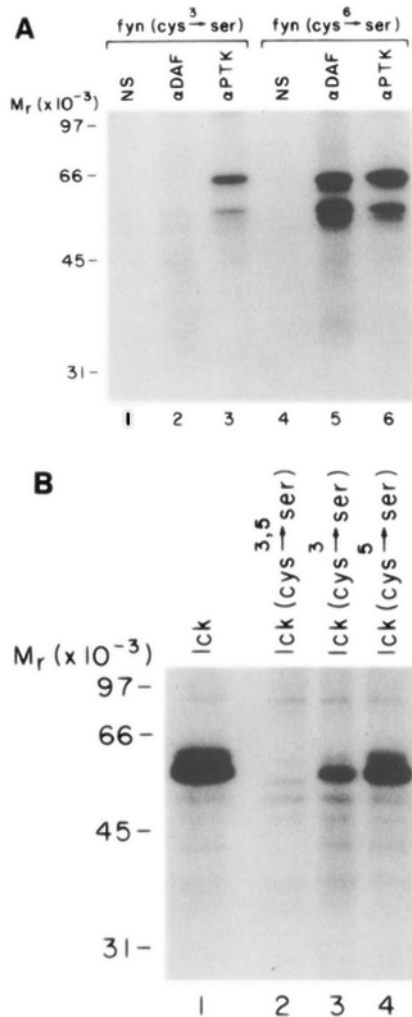


Figure 4. Association of DAF with PTK depends principally on Cys³ of PTK. (A) HeLa cells were transiently transfected with p59^{fyn} mutants with substitution of cysteine to serine at position 3 (lanes 1–3) or position 6 (lanes 4–6). Cell lysates were analyzed by immunoprecipitation with anti-DAF (lanes 2 and 5), anti-p59^{fyn} (lanes 3 and 6), or a nonspecific control antibody (lanes 1 and 4), followed by an *in vitro* kinase assay, SDS-PAGE, and autoradiography. The identity of the second higher relative molecular mass band is not known, but this has been observed previously with src family PTKs (38) and likely represents phosphorylation of serine residues (44), although coprecipitating proteins cannot be ruled out. In any case, this does not affect the conclusion that Cys³ of p59^{fyn} is the critical residue. (B) HeLa cells were transiently transfected with wild-type p59^{lck} (lane 1) or mutants with substitution of cysteine to serine at positions 3 and 5 (lane 2), position 3 alone (lane 3), or position 5 alone (lane 4). Cell lysates were subjected to anti-DAF immunoprecipitation and *in vitro* kinase assay as described above. Immunoprecipitation with anti-p59^{lck} showed approximately equal expression of p59^{lck} (data not shown).

p59^{fyn} (Fig. 7) into caveolae based on analysis of cell lysates. To directly demonstrate the colocalization of these proteins in cells, dual-color immunofluorescence was used. HeLa cells transfected with wild-type p59^{fyn} or p59^{fyn} mutants were permeabilized and stained with rabbit anti-p59^{fyn} and murine anti-DAF, followed by second antibodies against rabbit and murine IgG with distinct fluorescent labels. Dual

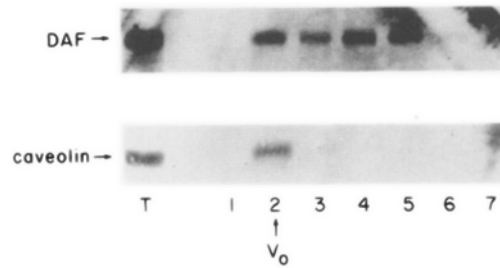


Figure 5. Triton-insoluble complexes contain caveolin. MDCK transfectants stably expressing human DAF were analyzed by chromatography of Triton X-100 cell lysates on Sepharose 4B columns as described in Materials and Methods. An equivalent aliquot of the total cell lysate (T) and column fractions (1–7) were Western blotted with either anti-DAF (above) or anticaveolin (below). V₀ designates the void volume of the column as marked by intact erythrocytes.

fluorescence microscopy demonstrated an overlapping punctate distribution of DAF and p59^{fyn} on the cell membrane (Fig. 10). Controls included mock-transfected cells to show the very low level endogenous p59^{fyn} expression, and HeLa cells transfected with the p59^{fyn}(Gly²→Ala) mutant to show the cytoplasmic expression of this nonacylated variant of p59^{fyn} (Fig. 10). Similar results were obtained for p56^{lck} (data not shown).

Palmitoylation of p59^{fyn} Moderately Increases its Membrane Avidity

Myristoylation of the Gly² residue of Src family PTKs is necessary for membrane binding (6, 9). It is possible that the

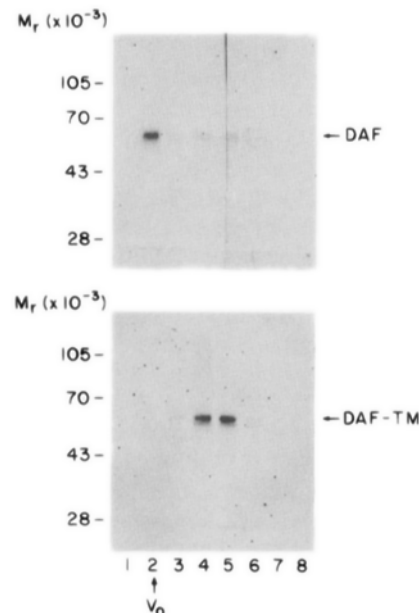


Figure 6. Inclusion of DAF in caveolae requires GPI anchor. Clones of EL-4 cells stably expressing equal levels of human DAF (wild-type GPI-anchored) or a chimeric transmembrane DAF molecule (DAFTM) were analyzed by chromatography of cell lysates on Sepharose 4B columns as described in Materials and Methods, followed by Western blotting of the column fractions with anti-DAF.

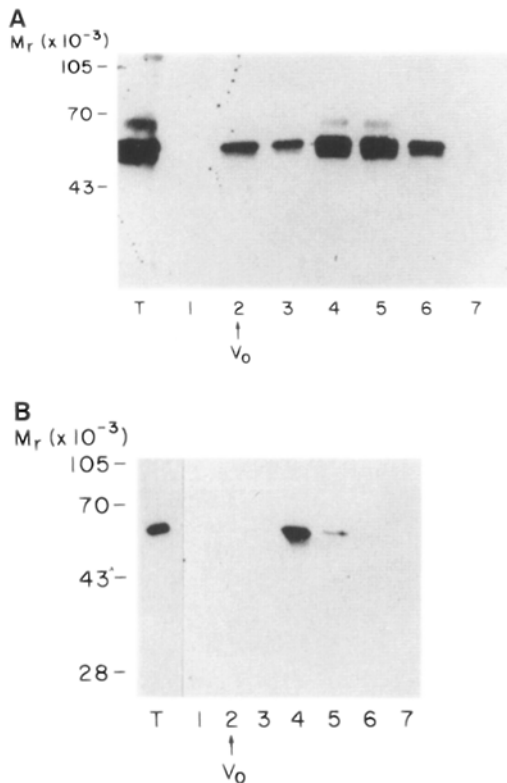


Figure 7. Inclusion of p59^{fyn} but not p60^{src} in caveolae. HeLa cells transiently transfected with (A) p59^{fyn} or (B) p60^{src} were analyzed by chromatography of cell lysates on Sepharose 4B columns as described in Materials and Methods, followed by Western blotting of the column fractions with antibody to the respective PTK. T represents an equal aliquot of the total cell lysate. V₀ marks the void volume of the column and the elution position of Triton-insoluble caveolae.

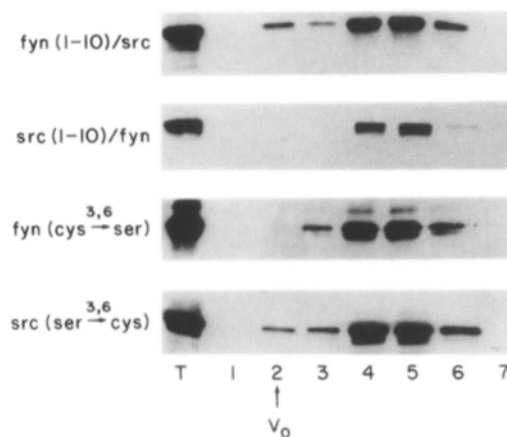


Figure 8. Inclusion of Src family PTK in caveolae maps to cysteine motif in first 10 amino acids. HeLa cells transiently transfected with the designated chimeric or mutant PTK were analyzed by Sepharose 4B chromatography followed by Western blotting with antibody to the appropriate PTK. fyn(1-10)/src designates a chimera with the first 10 amino acids of p59^{fyn} replacing the corresponding amino acid in p60^{src}, and src(1-10)/fyn designates the converse chimera. Only the section of the Western blot at the relative molecular mass of the PTK is shown (the rest of the blot was blank; see Fig. 7 for example).

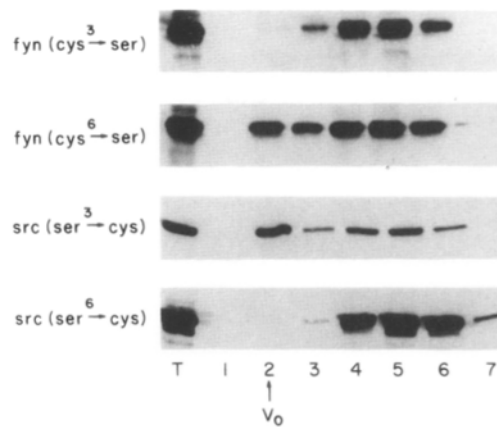


Figure 9. Inclusion of Src family PTK in caveolae maps to presence of Cys³ residue. HeLa cells transiently transfected with the designated mutants of p59^{fyn} or p60^{src} containing single substitution of cysteine and serine at positions 3 or 6 were analyzed by Sepharose 4B chromatography followed by Western blotting with antibodies to p59^{fyn} or p60^{src}.

presence of a second fatty acid on the PTK could influence the distribution of the protein between cytosol and plasma membrane. To explore this hypothesis, wild-type and mutant versions of p59^{fyn}, differing in their acylation patterns, were expressed in HeLa cells and analyzed for their membrane association. p59^{fyn} and the p59^{fyn}(Cys⁶→Ser) mutant, both of which are palmitoylated and myristoylated, were almost completely membrane bound (Fig. 11, lanes 1–2 and 7–8), whereas a p59^{fyn}(Gly²→Ala) mutant, which is neither myristoylated or palmitoylated, was almost exclusively cytosolic (Fig. 11, lanes 9 and 10). Prevention of palmitoylation (but not myristoylation) of p59^{fyn} by substitution of Cys³, alone or in combination with Cys⁶, reduced the percentage of p59^{fyn} that was membrane associated to a level intermediate between the wild-type and nonacylated protein (Fig. 11, lanes 3–6), suggesting a role for palmitate in strengthening membrane association. This conclusion must be made cautiously, however, since the stoichiometry of acylation of p59^{fyn} cannot be directly determined from these experiments.

Palmitoylation of p55^{fr} and p56^{hck}

To extend the findings on palmitoylation of Src family PTKs, two additional members of this group were studied. p55^{fr} and p56^{hck} are predominantly expressed in myeloid tissues. p55^{fr} has cysteine residues at positions 3 and 6, and p56^{hck} has a single amino-terminal cysteine residue at position 3. Based on our results on p59^{fyn} and p56^{hck}, these two additional PTKs would be expected to be modified by palmitoylation of Cys³, and furthermore, this should lead to interaction of the PTK with GPI-anchored proteins. To directly test this hypothesis, p55^{fr} and p56^{hck} were each expressed by transient transfection in HeLa cells. Analysis of complex formation between the PTK and DAF was done by immunoprecipitation of DAF and radiolabeling by an *in vitro* kinase reaction, and this demonstrated that both p55^{fr} and p56^{hck} were associated with DAF (Fig. 12 A). Radiolabeling with [³H]palmitate or [³H]myristate was next used to directly

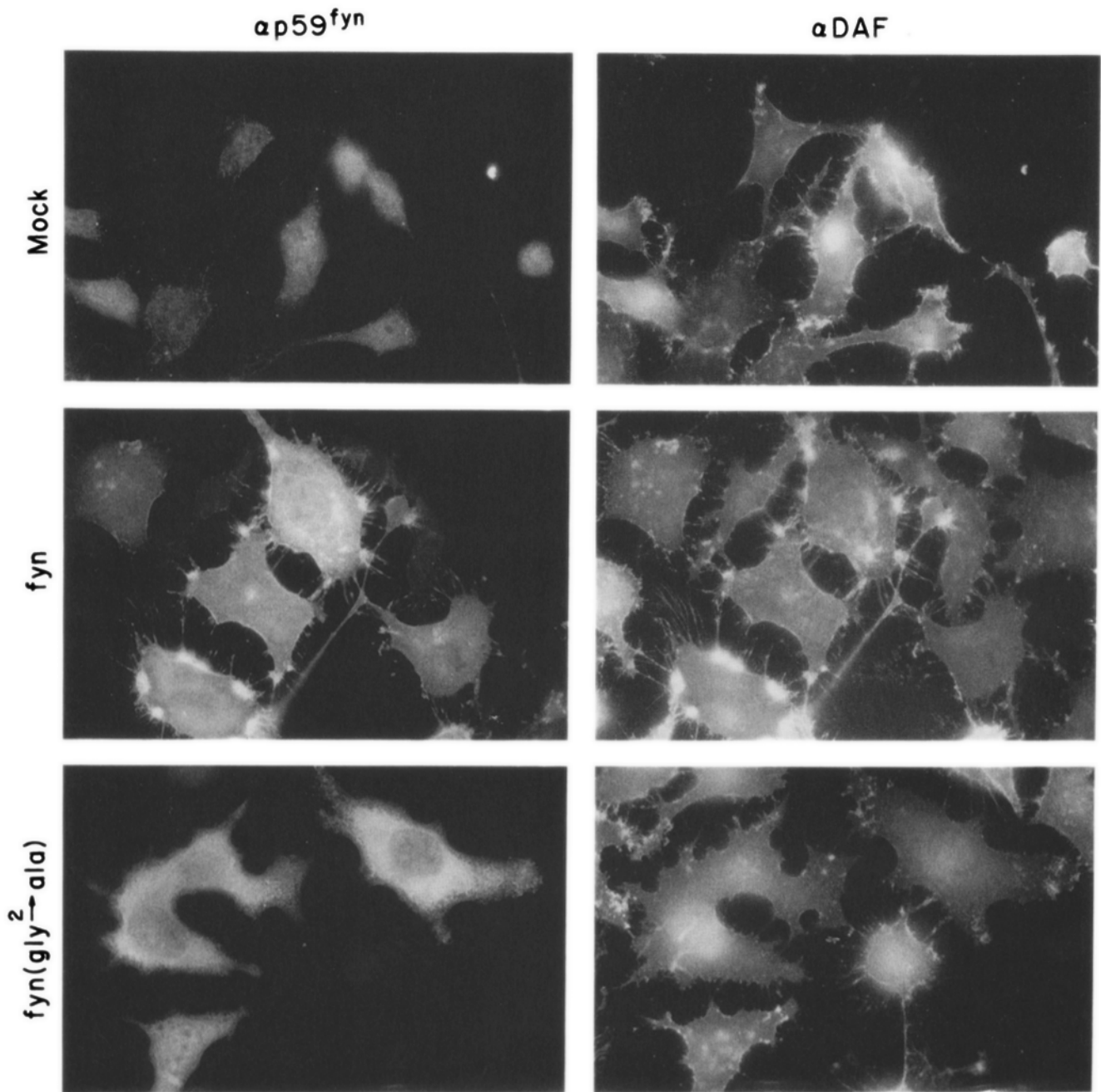


Figure 10. Colocalization of DAF and p59^{fyn} in cells by immunofluorescence. HeLa cells transiently transfected with p59^{fyn} (*middle row*), p59^{fyn}(Gly²→Ala) mutant (*bottom row*), or mock-transfected controls (*top row*), were permeabilized and stained with murine anti-DAF and rabbit anti-p59^{fyn}, followed by appropriate second antibodies with different fluorophores. Each field was photographed under the appropriate excitation light and filter to separately detect p59^{fyn} (left column) and DAF (right column). HeLa cells endogenously express DAF but are being transiently transfected with p59^{fyn}, so all cells in a field express DAF but only some express the transfected p59^{fyn}.

demonstrate that these PTKs were dually acylated with both fatty acids (Fig. 12 *B*). Treatment of the samples with hydroxylamine confirmed the distinction between the two fatty acids because only the [³H]palmitate label was removed by treatment with neutral hydroxylamine (Fig. 12 *B*).

Discussion

All the members of the Src family of PTKs are modified by

myristoylation of the Gly² residue through an amide linkage (3). Geahlen and co-workers recently demonstrated that p56^{lck} was also modified by palmitoylation (24), and our laboratory confirmed that finding and mapped the palmitoylation to an amino-terminal cysteine motif of p56^{lck} (38). In the present report, we have extended these results to three additional members of the Src family of PTKs by demonstrating palmitoylation of p59^{fyn}, p55^{lck}, and p56^{lck}. This palmitoylation occurred mainly, if not solely, on Cys³, and

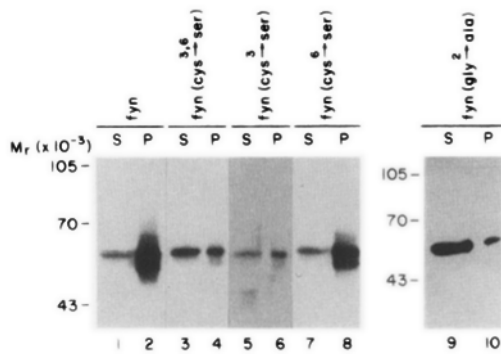


Figure 11. Membrane localization of wild-type and mutant p59^{fyn}. HeLa cells were transiently transfected with wild-type or mutant p59^{fyn} as designated. Cells were separated into supernatant (S) and pellet (P), representing cytosol and membrane fractions, respectively, and subjected to SDS-PAGE and Western blotting with anti-p59^{fyn}. To check that the high-level recombinant vaccinia expression system did not alter the membrane localization of p59^{fyn}, HeLa cell lines stably transfected with p59^{fyn} or p59^{fyn}(Gly²→Ala) were also analyzed with identical results (data not shown).

this defined an important amino-terminal peptide motif of Met-Gly-Cys for dual acylation of the PTK. Furthermore, important correlates of palmitoylation of this amino-terminal motif of Src family PTKs (which is not present in p60^{src}) were found to be localization of the protein into caveolae and a modest increase in overall membrane association.

The PTKs p56^{lck}, p59^{fyn}, p55^{fgr}, and p56^{hck} could incorporate [³H]palmitate as well as [³H]myristate. Only the [³H]palmitate was sensitive to removal by treatment with neutral hydroxylamine, indicating a thioester linkage, and the chemical identity of the bound radiolabel was confirmed by removal by alkaline hydrolysis and analysis by thin-layer chromatography. The thioester coupling of the palmitate suggests a linkage to cysteine, and the palmitoylation required Cys³ in p59^{fyn}, and Cys³ or Cys⁵ in p56^{lck}; substitution of these cysteine residues with serine blocked palmitoylation while leaving myristoylation intact. Additionally, introduction of Cys³ into the nonpalmitoylated p60^{src} led to its incorporation of [³H]palmitate. In the case of p56^{lck}, one cannot determine from these results whether Cys⁵ is a site of palmitoylation in the wild-type PTK, as it is possible that Cys⁵ is only palmitoylated in the absence of a preferred site at Cys³. In contrast, a single cysteine residue at position 6 could not support palmitoylation of p59^{fyn}. This could be caused by the slightly greater distance of the cysteine from the inner face of the plasma membrane, yet cysteine residues at positions 9 and 10 of the heterotrimeric guanine nucleotide-binding protein (G protein) α_q subunit have been implicated as the sites of palmitoylation (45). A more striking difference between the second cysteine residues in p59^{fyn} and p56^{lck} is the sequence following the amino acid: Lys-Asp in p59^{fyn} and Ser-Ser in p56^{lck}. The consensus sequence for palmitoylation of a cysteine residue is not well delineated, but this sequence difference between p56^{lck} and p59^{fyn} could account for the differential palmitoylation of the distal cysteine residue in these two PTKs.

In addition to the cysteine residue at position 3, palmitoy-

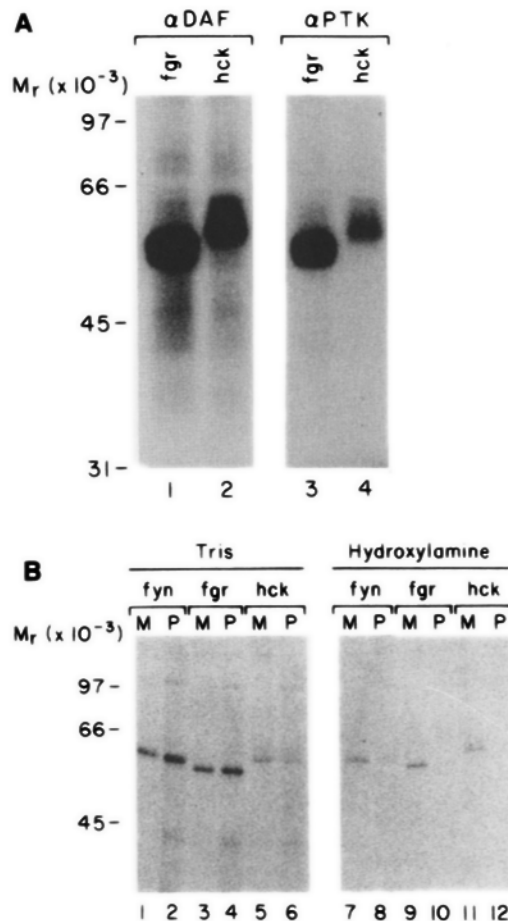


Figure 12. p55^{fgr} and p56^{hck} both associate with DAF and incorporate [³H]palmitate. (A) HeLa cells were transiently transfected with p55^{fgr} (lanes 1 and 3) or p56^{hck} (lanes 2 and 4), and nine tenths of the cell lysate was subjected to immunoprecipitation with anti-DAF (lanes 1 and 2), while one tenth of the cell lysate was immunoprecipitated with the appropriate anti-PTK (lanes 3 and 4). The immunoprecipitates were subjected to an *in vitro* kinase reaction followed by SDS-PAGE and autoradiography. Sequential immunoprecipitation with the respective anti-PTK of the *in vitro* kinase reaction product from the DAF immunoprecipitate was used to confirm the identity of p55^{fgr} and p56^{hck} (data not shown). (B) HeLa cells transiently transfected with p59^{fyn} (lanes 1, 2, 7, and 8), p55^{fgr} (lanes 3, 4, 9, and 10), or p56^{hck} (lanes 5, 6, 11, and 12) were biosynthetically labeled with [³H]myristate (M, odd-numbered lanes) or [³H]palmitate (P, even-numbered lanes) and analyzed by immunoprecipitation of the respective PTK from cell lysates. The immunoprecipitates were divided in half and run in duplicate lanes of SDS-PAGE. One set of lanes was treated with 1 M Tris-HCl, pH 7.5 (lanes 1-6), and the duplicate set was treated with 1 M hydroxylamine, pH 7.5 (lanes 7-12), followed by fluorography.

lation of the PTKs requires myristoylation. The Cys→Ser substitutions at the amino terminus of the PTKs did not block myristoylation. However, mutants of p56^{lck} and p59^{fyn} with a Gly²→Ala substitution, in which removal of the required glycine attachment site completely prevents myristoylation, are not palmitoylated. Without myristoylation of the PTK to provide membrane attachment, the protein might not be accessible to the palmitoyl transferase enzyme or palmitoyl-CoA. The stoichiometry of the acylation of the PTKs has not

been defined, so we cannot state what percentage of the protein molecules are dually acylated.

Data in this report have thus defined the peptide motif Met-Gly-Cys at the amino terminus of the protein as part of a consensus signal for dual acylation of members of the Src family of PTKs by myristate and palmitate. Several additional amino acid residues must form part of the signal because the myristoylation signal requires at least seven residues (15) and the palmitoylation consensus signal has yet to be well defined. Recent work by several groups has demonstrated the role of this Met-Gly-Cys motif in palmitoylation or dual acylation of the G protein α subunits (10, 19, 23, 25). Thus, this motif has been identified by parallel work on these two important classes of molecules, the Src family of PTKs and the G proteins, and it appears to serve a broad role in lipid modification of proteins.

Having established the dual acylation that arises from the Met-Gly-Cys motif, we went on to determine its effect on the localization of the PTK. Our previous work had established that removal of this motif abrogated the association between the PTK and the GPI-anchored proteins such as DAF (38). The present results demonstrate that this is caused by a requirement for this motif in partitioning of the PTK into caveolae, as assayed by the maintenance of these structures in the detergent Triton X-100. By analysis of chimeras between p59^{bn}, which is found in caveolae, and p60^{src}, which is not found in caveolae, we mapped the signal for inclusion of the PTK in caveolae to the Met-Gly-Cys motif. The Cys³ residue was necessary and sufficient within the context of a general Src family PTK to lead to inclusion in the caveolae because p59^{bn}(Cys³→Ser) lost its inclusion in caveolae, whereas p60^{src}(Ser³→Cys) was located in caveolae. Recent work has provided evidence for the presence of certain G protein α subunits in caveolae (35), and taken together with the presence of the Met-Gly-Cys motif in these proteins, we hypothesize that palmitoylation of this motif will be necessary for the inclusion of G proteins into caveolae. However, palmitoylation is not by itself sufficient for partitioning into caveolae because a chimeric type I transmembrane protein consisting of the extracellular portion of DAF, the transmembrane portion of an HLA class I molecule, and the cytoplasmic portion of p59^{bn} is palmitoylated but does not partition into caveolae (Kwong, J., D. J. Dietzen, and D. M. Lublin, unpublished observation). Relatively few proteins are present in caveolae (12), and there might be different signals for their inclusion. In this report, we have demonstrated that the GPI anchor and dual amino-terminal acylation of the Met-Gly-Cys motif are two such signals; because neither of these is present in caveolin, it must possess a distinct signal, and it is notable that caveolin has an unusually long putative transmembrane domain of 33 amino acids (14, 18). Further work will be needed to characterize the full range of signals for inclusion in caveolae.

The presence of Cys³, which leads to palmitoylation of the Src family PTKs, not only determines their localization in caveolae, but also appears to modestly strengthen their association with the plasma membrane. Because our experiments do not separately quantitate and analyze dual, single, and nonacylated molecules, any conclusions concerning the role of palmitoylation in membrane association must be tentative. Palmitoylation could lead to increased membrane association through a stronger binding to membrane lipids (or proteins) or through the additional protein fraction that is present in caveolae. It is also important to recognize that acy-

lation of the PTK is not necessarily the only factor in membrane binding. For example, p60^{src} is not modified by palmitoylation, yet other peptide sequences such as clusters of basic residues (39), together with myristoylation, have been implicated in directing membrane association of p60^{src} (16, 27, 39).

The palmitoylation of the Met-Gly-Cys motif can contribute to functional properties of the protein, either directly through inclusion in caveolae or by other effects on the protein. Our work in this area began with an investigation of the mechanism of signal transduction through crosslinking GPI-anchored proteins (37), and the present findings support a model of this signal transduction that is based on colocalization in caveolae of the GPI-anchored protein and Src family PTKs containing the Met-Gly-Cys motif. Indeed, crosslinking of the GPI-anchored protein Thy-1 on a murine T cell clone produced increased enzymatic activity of p56^{lck} (26). Similarly, we have found that crosslinking of DAF expressed in murine thymoma EL-4 cells leads within seconds to increased enzymatic activity of p56^{lck} and p59^{bn} (Kwong, J., and D. M. Lublin, unpublished observation), indicating the direct involvement of the PTKs in this signaling pathway. Since the GPI-anchored protein is inserted in the outer leaflet of the plasma membrane and the PTK is inserted in the inner leaflet, one must invoke an additional transmembrane linker protein to transduce signals between these proteins. Alternatively, a novel lipid-based property of the caveolae might be the basis for signal transduction. The detailed molecular mechanism coupling the GPI-anchored protein and the PTK within the caveolae remains to be elucidated. In addition to signal transduction, several other functions have been ascribed to caveolae, including internalization of small molecules and ions by potocytosis (2), and acting to sort GPI-anchored proteins to the apical surface in polarized epithelium (4, 12, 20, 35). The possible role of palmitoylated Src family PTKs (or G protein α subunits) in these functions deserves study.

In addition to its role in directing Src family PTKs into caveolae, the palmitoylation of the amino-terminal cysteine motif could have other functional consequences. Palmitoylation is a reversible process, and this has been directly demonstrated for palmitoylation of p56^{lck} (24). This raises the possibility of regulation of signal transduction by a process of palmitoylation and depalmitoylation. Recent evidence has shown an increase in palmitoylation of G protein α subunits upon activation with the β -adrenergic receptor agonist isoproterenol (11, 23) with pulse-chase experiments indicating an increased turnover of palmitate in α , after isoproterenol treatment (23). Furthermore, palmitoylation of G protein α subunits is required for signaling by the α , and α_q subunits (45). Changes in palmitoylation of Src family PTKs upon activation have not yet been investigated. Palmitoylation of Src family PTKs could also influence other functions of these proteins. For example, p59^{bn} and p56^{lck} are implicated in signaling through the T cell receptor (17), but it is not known if palmitoylation of these PTKs is involved. For the p59^{bn} interaction with the CD3/ ζ chain complex, the association has been mapped to the 10 amino-terminal amino acids of p59^{bn} (42), and thus the Met-Gly-Cys motif could be a factor. However, p56^{lck} possesses the same motif yet cannot substitute for p59^{bn} in associating with the CD3/ ζ chain complex, suggesting that some separate or additional factor must also be involved in this interaction.

Overall, we have now defined the molecular basis of inclu-

sion of Src family PTKs in caveolae through dual acylation of the amino-terminal motif Met-Gly-Cys. The elucidation of the roles of these PTKs and the G protein α subunits that share this motif in the structure and function of the caveolae should prove to be an area of fruitful investigation.

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