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Signals Determining Protein Tyrosine Kinase and Glycosyl-Phosphatidylinositol-Anchored Protein Targeting to a Glycolipid-Enriched Membrane Fraction

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Glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins and certain protein tyrosine kinases associate with a Triton X-100-insoluble, glycolipid-enriched membrane fraction in MDCK cells. Also, certain protein tyrosine kinases have been shown to associate with GPI-anchored proteins in other cell types. To characterize the interaction between GPI-anchored proteins and protein tyrosine kinases, GPI-anchored proteins were coexpressed with $p56^{lck}$ in HeLa cells. Both proteins were shown to target independently to the glycolipid-enriched membranes. Coimmunoprecipitation of GPI-anchored proteins and $p56^{lck}$ occurred only when both proteins were located in the glycolipid-enriched membranes, and gentle disruption of these membranes abolished the interaction. The GPI anchor was found to be the targeting signal for this membrane fraction in GPI-anchored proteins. Analysis of mutants indicated that $p56^{lck}$ was nearly quantitatively palmitoylated at Cys-5 but not palmitoylated at Cys-3. The nonpalmitoylated cysteine at position 3 was very important for association of $p56^{lck}$ with the membrane fraction, while palmitoylation at Cys-5 promoted only a low level of interaction. Because other *src* family protein tyrosine kinases that are associated with GPI-anchored proteins always contain a Cys-3, we propose that this residue, in addition to the N-terminal myristate, is part of a common signal targeting these proteins to a membrane domain that has been linked to transmembrane signaling.

Proteins that are anchored to membranes by conjugation to glycosyl-phosphatidylinositol (GPI anchored) (see reference 13 for a review) exhibit interesting properties. For example, cross-linking of GPI-anchored proteins on the surface of T cells results in activation (10, 24, 25). In epithelial cells, the GPI anchor also serves as a sorting signal (6, 22, 32, 55) directing proteins to specific cell surfaces. Recent characterization of GPI-anchored proteins in MDCK cells has shown that they associate in the Golgi apparatus and at the cell surface with a low-density membrane fraction that is enriched in sphingolipids, including glycolipids, and is resistant to solubilization by nonionic detergents (5). We will call this the glycolipid-enriched membrane (GEM) fraction.

A complex containing caveolin and other proteins is also contained within the GEM fraction in MDCK cells (23, 41). Because caveolin is a major protein found in small invaginations called caveolae at the cell surface (38), it is likely that the GEM fraction includes partially purified caveolae. A similar low-density Triton X-100 (TX100)-insoluble fraction has been described for Rat-1 fibroblasts and Caco cells (15, 41).

Several studies have shown association of protein tyrosine kinase activity with GPI-anchored proteins in nonionic detergent lysates of cells (12, 45, 46, 48, 49), but the nature of the association has not been established. This association has been observed for both lymphoid and HeLa cells with the nonreceptor protein tyrosine kinases $p56^{lck}$, $p59^{fyn}$, and $p56^{lyn}$. In experiments showing association of GPI-anchored proteins and protein tyrosine kinases, the experimental conditions were those that should maintain the GEM fraction characterized by Brown and Rose (5), i.e., cell lysis in nonionic detergent at 4°C. Other studies have shown the presence of large detergent-resistant complexes containing GPI-anchored proteins and protein tyrosine kinases in lymphoid cells (3, 7, 12). These complexes likely represent the same glycolipid-enriched membranes characterized previously, but the lipid composition has not been reported.

To characterize the interaction between GPI-anchored proteins and $p56^{lck}$ in the GEM fraction, these proteins were transiently expressed in HeLa cells and the GEM fraction was isolated by equilibrium centrifugation. Expression of different protein constructs and subsequent isolation of the GEM fraction have allowed us to define the signals mediating targeting of each protein class to this fraction.

MATERIALS AND METHODS

Materials. Trans ^{35}S-label, [^{3}H]myristate, and [^{3}H]palmitate were purchased from DuPont (Wilmington, Del.). Rabbit antiserum to placental alkaline phosphatase (PLAP) was purchased from Dako Corp. (Carpinteria, Calif.), and Pansorbin was purchased from Calbiochem (San Diego, Calif.). Polyclonal rabbit antiserum to vesicular stomatitis virus (VSV) and a rabbit antipeptide serum to $p56^{lck}$ were prepared as described elsewhere (36, 43).

Cell culture and protein expression. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were maintained at 37°C in the presence of 5% CO_2.

Protein expression was achieved by infection of HeLa cells (4×10^5 cells per 35-mm-diameter dish) with the recombinant vaccinia virus vTF7-3 encoding the T7 RNA polymerase (14), followed by transfection with plasmid DNA. The cells were infected with vaccinia virus at a multiplicity of infection of 20 to 30 in 0.5 ml of DMEM. Following incubation with the virus at 37°C for 30 min, the inoculum was removed and the cells were transfected with 5 μg of DNA and 15 μl of cationic liposomes (37) in 1.5 ml of DMEM.

The cells were metabolically labeled at 3 h posttransfection.

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For [^{35}S]methionine labeling, the cells were first washed and incubated in DMEM minus methionine for 15 min at 37°C. The cells were then incubated for 1 h at 37°C in 1 ml of DMEM minus methionine containing 100 μCi of [^{35}S]methionine. Protein labeling with [^{3}H]myristate and [^{3}H]palmitate was done following washing and incubation of the cells in fatty acid labeling medium consisting of DMEM containing 10% tryptose phosphate buffer, sodium pyruvate (1.0 mM), and nonessential amino acids (1.0 mM). The cells were then incubated for 90 min at 37°C with [^{3}H]myristate (200 μCi/ml) or [^{3}H]palmitate (400 μCi/ml) in 1 ml of fatty acid labeling medium. Prior to addition to the labeling medium, the ^{3}H-fatty acids were dried and dissolved in dimethyl sulfoxide in a volume equaling 1% of the total labeling volume. After protein labeling, the cells were lysed either immediately or following a chase period in DMEM supplemented with 10% fetal bovine serum and 2 mM unlabeled methionine.

Construction of mutants. Construction of plasmids encoding p56^{lck} (pGEM-*lck*), the p56^{lck} mutant lacking the N-terminal myristoylation (G2A), GThy-1, and VSV G has been previously described (9, 43, 44, 53). pGEM-PLAP was generously provided by S. Udenfriend, and the orientation of the insert was reversed for transcriptional control by the T7 promoter. The chimeric protein PLAPG, consisting of the PLAP ectodomain with the VSV glycoprotein (G) transmembrane and cytoplasmic domains (see Fig. 1) (1), was inserted into pGEM-4 with transcriptional control by the T7 promoter by performing PCR (39) on the previously described construct containing PLAPG (6). The oligonucleotides used for the PCR were 5′-CGATCGAAGCTTCCCAGCATGCTGCTGCTGC TGCTG and 5′-ACGTGCAGATCTGCAGGATTTGAGTT ACTTTC. The underlined sequences are *Hin*dIII and *Bgl*II restriction sites introduced at the ends of the PCR product. The PCR fragment was digested with *Hin*dIII and *Bgl*II and cloned into *Hin*dIII- and *Bam*HI-digested pGEM-4. p56^{lck} mutants were generated by oligonucleotide-directed mutagenesis using PCR. Codons for Cys-3 and Cys-5 were changed to serine residues either individually or together, and the mutants are designated C3S; C5S; and C3,5S. The mutagenic oligonucleotides used for PCR were 5′-TAAGGAATTCATG GGCTCTGTCTGCAGC TCAAACCCTGAAG, 5′-GGAGG AATTCATGGGCTGTGTCTCCAGCTCAAACCCTGAAG, and 5′-TAAGGAATTCATGGGCTCTGTCTCCAGCTCAA ACCCTGAAG for the C3S, C5S, and C3,5S mutants, respectively. The PCR was conducted with each mutant primer in combination with the primer 5′-TTCCGGATGGGCAGC GAGATCT. The PCR fragments were digested with *Eco*RI and *Bgl*II and cloned into *Eco*RI- and *Bgl*II-digested pGEM-*lck*. All sequences generated by PCR were confirmed by DNA sequencing (40).

Cell lysis and equilibrium centrifugation. The cells were first washed with phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate [pH 7.4]). For experiments employing equilibrium centrifugation, lysis was in 0.75 ml of 1% TX100–10 mM Tris-HCl (pH 7.5)–150 mM NaCl–5 mM EDTA–10 mM iodoacetamide–75 U of aprotinin. The lysate was collected with a rubber policeman and then Dounce homogenized (eight strokes) on ice. The lysate was then centrifuged for 5 min at 1,300 × *g* to remove the nuclei and other large cellular debris. All steps were performed at 4°C.

For equilibrium centrifugation, the clarified lysate was diluted with an equal volume of an 85% (wt/vol) sucrose solution in 10 mM Tris (pH 7.5)–150 mM NaCl–5 mM EDTA (TNE). The diluted lysate was placed at the bottom of a Beckman SW41 centrifuge tube, and 6.0 ml of a 30% (wt/vol) sucrose solution in TNE and then 3.5 ml of a 5% (wt/vol) sucrose solution in TNE were layered above it. The samples were centrifuged at 200,000 × *g* for 14 h. Following centrifugation, the gradients were fractionated by collection of the material from the top of the gradient. All steps were performed at 4°C.

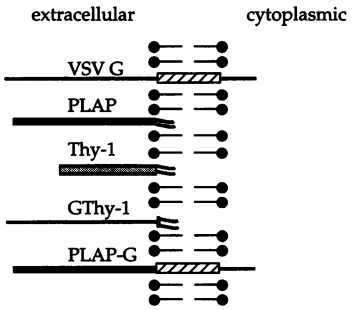

FIG. 1. Chimeric protein constructs. PLAP, Thy-1, and GThy-1 are proteins anchored to the membrane by the GPI moiety. PLAPG contains the transmembrane and cytoplasmic domains of VSV G with the PLAP ectodomain. GThy-1 contains the VSV G ectodomain anchored to the membrane by a GPI moiety.

For experiments examining total protein expression without gradient separation, the lysis was in 1.0 ml of a solution containing 1% Nonidet P-40–0.4% deoxycholate–66 mM EDTA–10 mM Tris (pH 7.4). The lysate was clarified by centrifugation for 5 min at 16,000 × *g* at 4°C.

Immunoprecipitations. Each gradient fraction was diluted with an equal volume of chilled 1% TX100 in TNE and precleared by incubating with 10 μl of Pansorbin for 1 h and then centrifuging (16,000 × *g* for 5 min). One microliter of rabbit antiserum to the corresponding protein was added to each precleared fraction, and the mixtures were incubated overnight. Ten microliters of Pansorbin was again added to each fraction, and the samples were incubated for an additional hour. The antibody-antigen complexes were pelleted by a 1-min centrifugation (16,000 × *g*), and the samples were washed twice with chilled 1% TX100 in TNE. All steps were performed at 4°C. The material was eluted into a sample buffer used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

When total protein expression was examined without gradient separation, SDS was added to the clarified lysate to a final concentration of 0.1%. The lysate was incubated with antiserum for 30 min at 37°C and then with Pansorbin for 15 min at 37°C. Following precipitation, the Pansorbin was washed with a 10 mM Tris–0.1% SDS–1% deoxycholate–1% Nonidet P-40–150 mM NaCl (pH 7.4) solution. The samples were then eluted in SDS-PAGE sample buffer.

SDS-PAGE was done by using Hoefer (San Francisco, Calif.) Mighty Small electrophoresis units with a 10% polyacrylamide gel (21). The gels were visualized by fluorography (4) and quantitated by densitometry (model GS 300; Hoefer).

RESULTS

GPI-anchored proteins are associated with a nonionic-detergent-insoluble fraction in HeLa cells. GPI-anchored proteins associate in MDCK cells with a GEM fraction that is resistant to solubilization by nonionic detergents (5). To determine if a TX100-resistant membrane fraction existed in HeLa cells, PLAP (Fig. 1), a GPI-anchored protein, was expressed by using the vaccinia virus-T7 system (see Materials and Methods). PLAP associates with the detergent-resistant membranes only after transport to the Golgi in MDCK cells

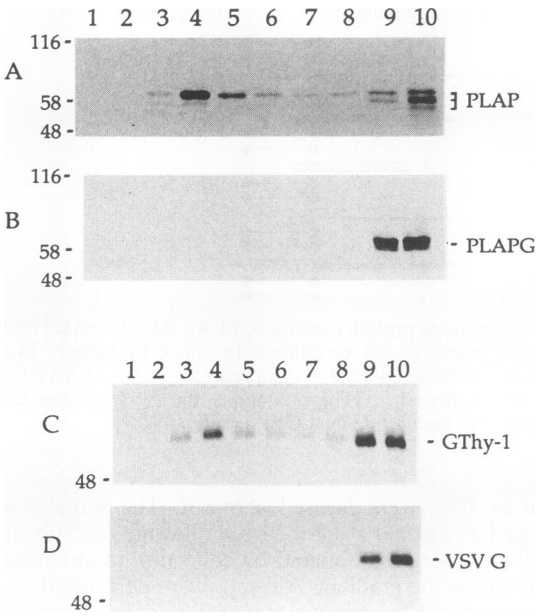

FIG. 2. Equilibrium centrifugation of TX100 lysates from cells expressing PLAP, PLAPG, GThy-1, or VSV G. HeLa cells were transfected with 5 μg plasmid DNA encoding PLAP (A), PLAPG (B), GThy-1 (C), or VSV G (D). Following labeling with [^{35}S]methionine and a 2-h chase, the cells were lysed with a 1% TX100 detergent solution. After equilibrium centrifugation in a discontinuous sucrose gradient, the gradients were fractionated and immunoprecipitated with rabbit antiserum to each of the proteins. Fraction 1 represents the top of the gradient. Fractions 3 to 5 were used to represent the amount of protein associated with the GEM fraction. Molecular weights (in thousands) are indicated on the left.

(5). Therefore, the cells were subjected to a 2-h chase following labeling to allow time for transport of the labeled protein from the endoplasmic reticulum. The TX100 lysate of the cells was then subjected to equilibrium density gradient centrifugation in a discontinuous sucrose gradient to separate the low-density detergent-resistant membrane fraction from the TX100-soluble material.

The TX100-resistant membrane fraction localized at the interface between the top (5%) and middle (30%) sucrose layers, corresponding to fractions 3 to 5 in Fig. 2. The TX100-soluble material remained at the bottom of the gradient in fractions 9 and 10. To determine if the detergent-resistant membranes of the HeLa cells were enriched in sphingolipids as described for MDCK cells (5), the lipids from detergent-resistant membranes were extracted (2) and separated by thin-layer chromatography using CH_3Cl-MeOH-NH_4OH (60:40:5). Using α-naphthol (51) for detection, we determined that the majority of the lipids from this fraction represented sphingolipids (data not shown). The detergent-resistant membranes of HeLa cells, therefore, also represent a GEM fraction.

Sixty-five percent of the labeled PLAP was associated with the GEM fraction (Fig. 2A). PLAP separates into three bands representing different extents of oligosaccharide processing. However, it is the upper band that is present in the GEM fraction. This band represents PLAP that has been transported to the Golgi and has processed oligosaccharides as described earlier (5).

To determine if association of PLAP with the GEM fraction was due to the GPI anchor, the chimeric protein PLAPG (1)

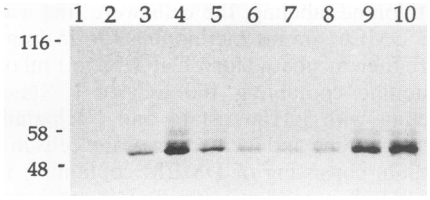

FIG. 3. Association of p56^{lck} with the GEM fraction in HeLa cells. HeLa cells were transfected with 5 μg of plasmid DNA encoding p56^{lck}. Protein labeling, cell lysis, and membrane flotation were done as outlined for Fig. 2. The resulting gradient fractions were immunoprecipitated with rabbit antiserum to p56^{lck}. Fraction 1 represents the top of the gradient. Fractions 3 to 5 were used to represent the amount of protein associated with the GEM fraction. Molecular weights (in thousands) are indicated on the left.

(see Materials and Methods) was expressed in HeLa cells. Following metabolic labeling and chase, a TX100 lysate of the cells was subjected to equilibrium centrifugation as described for PLAP. The results of the gradient are presented in Fig. 2B. All of the PLAPG was soluble in TX100, confirming that association of PLAP with the GEM fraction results from the presence of the GPI anchor. Since PLAPG was transported to the Golgi as rapidly as PLAP, the failure of PLAPG to associate with the GEM fraction was not due to a block in transport.

To extend the analysis to two other proteins, we compared the association of VSV G and GThy-1 with the GEM fraction. GThy-1 contains the VSV G ectodomain and the signal for GPI addition from Thy-1 (Fig. 1). Previous studies showed that GThy-1 is linked to the membrane by a GPI anchor (9). Figure 2C shows that 25% of the labeled GThy-1 associated with the GEM. Conversely, the membrane-spanning VSV G protein was entirely TX100 soluble as previously shown with MDCK cells (5). The smaller amount of GThy-1 association with the GEM fraction relative to PLAP probably results from the reduced efficiency of transport of this chimera (9).

Association of PLAP and p56^{lck} in the GEM fraction. The *src* family tyrosine kinase p56^{lck} is found in large complexes with GPI-anchored proteins in nonionic detergent lysates from lymphoid and HeLa cells (3, 7, 12, 46, 48). It seemed likely that these complexes might be the GEM fraction because the conditions that were used in those experiments would preserve interaction with the GEM fraction. To determine if p56^{lck} associated with the GEM fraction in HeLa cells, we expressed it and performed protein labeling, lysis, and equilibrium centrifugation as described above for the GPI-anchored proteins. Figure 3 shows that approximately 50% of the labeled p56^{lck} was associated with the GEM fraction.

To determine if PLAP and p56^{lck} coimmunoprecipitated in the GEM fraction, the proteins were coexpressed in HeLa cells. Following equilibrium centrifugation of the TX100 lysate, the gradients were fractionated. Half of each fraction was immunoprecipitated with anti-PLAP and half with anti-p56^{lck}. PLAP and p56^{lck} coimmunoprecipitated only in the GEM fractions (gradient fractions 3 to 5 in Fig. 4). We also observed coimmunoprecipitation of GThy-1 and p56^{lck} in the GEM fraction of cells coexpressing these proteins (data not shown). The absence of coimmunoprecipitation in the soluble fraction demonstrates that the coimmunoprecipitations in the GEM fraction are not due to cross-reactivity of the respective antibodies.

Because the GEM fraction contains large membrane sheets and vesicles (5, 41), we controlled for the possibility that the

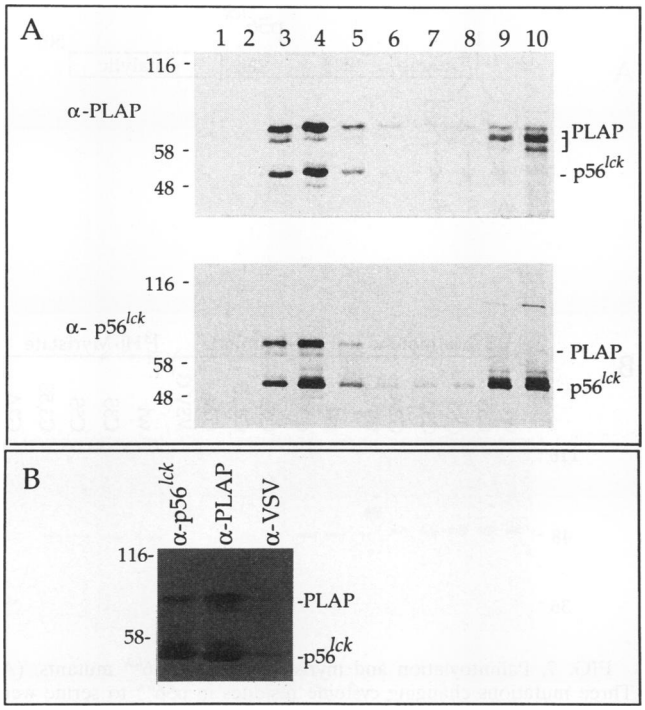

FIG. 4. Coimmunoprecipitation of PLAP and p56^{lck} in the GEM fraction. (A) HeLa cells were cotransfected with 1 μg of DNA encoding PLAP and 4 μg of DNA encoding p56^{lck}. The cells were labeled with [^{35}S]methionine, chased for 2 h, and lysed with a 1% TX100 solution. Following equilibrium centrifugation, each gradient fraction was immunoprecipitated with rabbit antiserum to PLAP (α-PLAP) or p56^{lck} (α-p56^{lck}). Fraction 1 represents the top of the gradient. (B) HeLa cells were cotransfected, labeled, and lysed as described for panel A. Following equilibrium centrifugation, gradient fractions 3 to 5 were collected and immunoprecipitated with antibody (α) to p56^{lck}, PLAP, or VSV G as an irrelevant antibody. Molecular weights (in thousands) are indicated on the left.

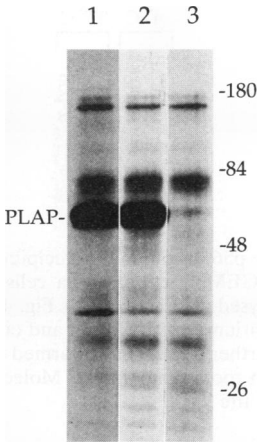

FIG. 5. Immunoprecipitation and sedimentation of the GEM fraction. HeLa cells were either transfected with PLAP (lanes 1 and 2) or mock transfected (lane 3) and prelabeled by adding [^{35}S]methionine at 2 h posttransfection. After 2 h of labeling and a 2-h chase, the cells were lysed and the GEM fraction was isolated as described for Fig. 4. The GEM fraction was then immunoprecipitated by adding antibody to PLAP (lane 1) or pelleted by centrifugation (lanes 2 and 3). For pelleting of the membranes, the GEM fraction was diluted 1:10 with 1% TX100 in TNE and centrifuged at 200,000 × *g* for 3 h at 4°C. Molecular weights (in thousands) are indicated on the right.

apparent coimmunoprecipitation of PLAP and p56^{lck} merely represented nonspecific sedimentation of membranes containing the proteins. Following equilibrium centrifugation of the TX100 lysate of cells cotransfected with PLAP and p56^{lck}, fractions 3 to 5 were collected, precleared, and incubated with antibody to PLAP or p56^{lck} or an irrelevant antibody. The sample incubated with the irrelevant antibody showed only a low level of precipitation of PLAP or p56^{lck}, less than 15% of the amount precipitated with PLAP or p56^{lck} antibody (Fig. 4B). Thus, only a small fraction of the material in the immunoprecipitations resulted from nonspecific pelleting of membranes. Furthermore, when PLAP and p56^{lck} were expressed separately and the cells were mixed prior to lysis, there was no coimmunoprecipitation of PLAP and p56^{lck} in the isolated GEM fraction (data not shown). The latter point shows that the GEM fraction does not represent a mixing artifact following detergent lysis.

Because the GEM fraction consists largely of membrane sheets and vesicles, we considered it likely that immunoprecipitation of PLAP or p56^{lck} resulted in precipitation of the entire GEM fraction. To examine this possibility, host proteins in cells transfected with DNA encoding PLAP were labeled for 2 h with [^{35}S]methionine starting at 2 h posttransfection. Labeling, therefore, began prior to shutoff of host protein synthesis by vaccinia virus. Following a 2-h chase, TX100 lysis, and equilibrium centrifugation, the GEM fraction (gradient fractions 3 to 5) was collected and either immunoprecipitated with antibody to PLAP or pelleted by centrifugation. The GEM fraction from nontransfected, vaccinia virus-infected cells was also prepared and pelleted. Figure 5 (lanes 1 and 2) shows PLAP and host protein bands with molecular weights of 180,000, 130,000, 80,000, 40,000, 35,000, and 28,000 that were reproducibly present in both the PLAP immunoprecipitate and the sediment of the GEM fraction. Two faint bands occur in the 22- to 24-kDa region, corresponding approximately to the size of caveolin, a protein which has been shown to associate with the GEM fraction in MDCK cells (41). The GEM fraction from cells infected with vaccinia virus but not transfected (lane 3) shows the same host cell proteins as the transfected cells. The proteins in the GEM fraction from uninfected cells appeared identical to those of the untransfected cells (data not shown).

Coimmunoprecipitation of PLAP and p56^{lck} requires an intact GEM fraction. To determine if the GEM fraction must be intact for coimmunoprecipitation of PLAP and p56^{lck}, cells expressing both proteins were metabolically labeled and the GEM fraction was isolated by equilibrium centrifugation. Because earlier studies showed that the GEM fraction was solubilized in TX100 at 37°C (5), one half of the sample was immunoprecipitated at room temperature following a 5-min incubation at 37°C. The second half of the sample was immunoprecipitated at 4°C with no incubation at 37°C. As shown in Fig. 6, when the sample was warmed to 37°C, no coimmunoprecipitation of PLAP and p56^{lck} occurred. The observation that the membranes must remain intact for the coimmunoprecipitation to occur suggests that PLAP and p56^{lck} are not directly associated but simply anchored in the same membrane.

Targeting signal in p56^{lck}. Recent studies have shown that p56^{lck} is palmitoylated on one or more cysteines in addition to having an N-terminal myristate group (30). From analysis of a single p56^{lck} mutant lacking both N-terminal cysteines 3 and 5, Shenoy-Scaria et al. (46) suggested that both of these cysteines

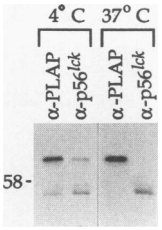

FIG. 6. PLAP and $p56^{lck}$ coimmunoprecipitation is abolished by solubilization of the GEM fraction. HeLa cells were cotransfected, labeled, chased, and lysed as described for Fig. 4. The GEM fraction was isolated by equilibrium centrifugation and either immunoprecipitated at 4°C with no further treatment or warmed to 37°C for 5 min and immunoprecipitated at room temperature. Molecular weight (in thousands) is indicated on the left.

were palmitoylated. Because *src* kinases lacking these N-terminal cysteines are not in a complex with GPI-anchored proteins (12), we considered the possibility that palmitoylation at cysteine 3 or 5 might signal association with the GEM fraction. To test this, we prepared mutants C3S, C5S, and C3,5S (Fig. 7A), lacking each or both of these cysteines and examined their palmitoylation. We also examined the mutant G2A (Fig. 7A), which cannot be myristoylated since it does not have the required N-terminal glycine (18).

Metabolic labeling of each of the $p56^{lck}$ mutants with either [^{35}S]methionine or [^{3}H]palmitate is illustrated in Fig. 7B. The [^{35}S]methionine label showed that the mutants and wild-type $p56^{lck}$ were expressed at similar levels. While both the wild type and the C3S mutant demonstrated comparable levels of [^{3}H]palmitate labeling, the C5S and C3,5S mutants were labeled at a level of about 25% of that measured for the wild-type and C3S proteins. The similar low levels of ^{3}H labeling for both C5S and C3,5S indicate that only the cysteine at position 5 is palmitoylated.

The G2A myristoylation mutant showed no labeling with [^{3}H]palmitate (Fig. 7B). Since this mutant did not bind to the membrane, palmitoylation by a membrane-bound palmitoyltransferase was probably prevented (18). The complete lack of labeling of the G2A mutant demonstrates that the residual labeling of the C5S and C3,5S mutants was not a result of metabolic degradation of [^{3}H]palmitate and reincorporation into protein as ^{3}H-labeled amino acids. Thus, the small amount of ^{3}H labeling of C5S and C3,5S probably results from degradation of [^{3}H]palmitate to [^{3}H]myristate. Alternatively, there could be a low level of palmitoylation elsewhere in $p56^{lck}$.

To quantitate the number of palmitates added to $p56^{lck}$, the VSV G protein (Fig. 1) was expressed and labeled with [^{3}H]palmitate as an internal standard since it has been shown previously to contain a single palmitate molecule attached to a cysteine residue in the cytoplasmic domain (35, 42). By comparing the ratios of ^{3}H labeling to ^{35}S labeling in VSV G and $p56^{lck}$, including subtracting the background labeling of the C3,5S mutant, we calculated that $p56^{lck}$ is 90% palmitoylated at Cys-5. This calculation also corrected for the number of methionines in the two proteins (11 in $p56^{lck}$ and 10 in VSV G). Because cysteines near the C termini of proteins are known to be isoprenylated (8), we considered the possibility that Cys-3 might have some novel type of isoprenylation. However, attempts to label $p56^{lck}$ with [^{3}H]mevalonate, a precursor of isoprenyl groups, were unsuccessful.

To determine if the signal for myristoylation was disrupted in any of the cysteine mutants, we also labeled cells expressing wild-type $p56^{lck}$ or the C3S, C5S, C3,5S, and G2A mutants with

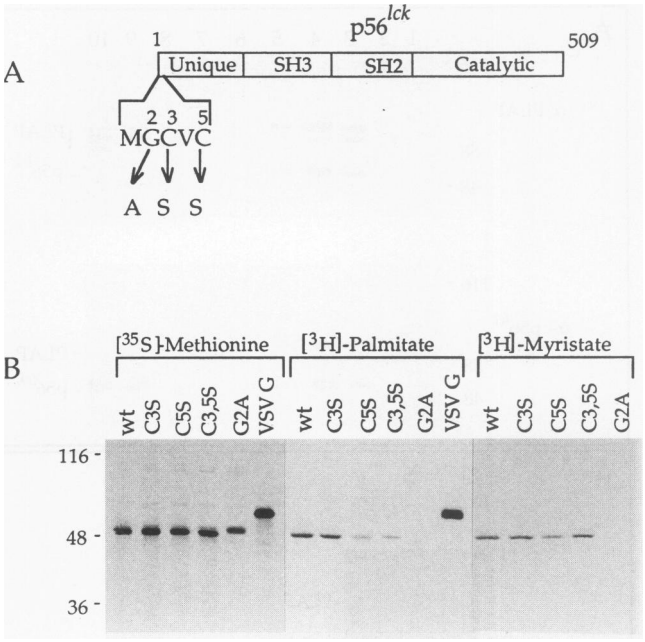

FIG. 7. Palmitoylation and myristoylation of $p56^{lck}$ mutants. (A) Three mutations changing cysteine residues in $p56^{lck}$ to serine were constructed. C3S and C5S have the cysteines at positions 3 and 5 replaced with serine, respectively. C3,5S has both cysteines at positions 3 and 5 replaced with serines. A $p56^{lck}$ mutant (G2A) with glycine replaced with alanine (41) was also employed. (B) Wild-type $p56^{lck}$ (wt) and each of the $p56^{lck}$ mutants were expressed in HeLa cells. Cells were metabolically labeled with either [^{35}S]methionine, [^{3}H]palmitate, or [^{3}H]myristate. Following labeling, the cells were lysed and the proteins were immunoprecipitated with rabbit antiserum to $p56^{lck}$. As a reference for quantitation of $p56^{lck}$ palmitoylation, VSV G was expressed and labeled with [^{35}S]methionine or [^{3}H]palmitate. Autoradiograms were quantitated by densitometry. The ratios of [^{3}H]palmitate labeling to [^{35}S]methionine labeling measured by densitometry were calculated to be 0.25, 0.21, 0.07, 0.08, and 0.21 for wild-type, C3S, C5S, and C3,5S $p56^{lck}$ molecules, respectively. The ratio for VSV G, measured at a lighter exposure, was 0.21. Molecular weights (in thousands) are indicated on the left.

[^{3}H]myristate (Fig. 7B). As shown in Fig. 7B, each of the cysteine-to-serine mutants had levels of myristate labeling equal to that for the wild-type $p56^{lck}$. As expected, the G2A mutant was not myristoylated because of the removal of the N terminal glycine.

To determine the amount of association of the $p56^{lck}$ cysteine mutants with the GEM fraction, each was expressed in HeLa cells and labeled with [^{35}S]methionine. Following the chase, lysis, and equilibrium centrifugation, the gradient fractions were immunoprecipitated with antiserum to $p56^{lck}$. The results of the experiment are shown in Fig. 8. Forty to forty-five percent of the labeled wild-type $p56^{lck}$ and C5S mutant was associated with the GEM fraction, while only 14% of the labeled C3S mutant and none of the C3,5S and G2A mutants were associated with the GEM fraction. Since the C5S mutant lacks the N-terminal palmitate, these data demonstrate that $p56^{lck}$ palmitoylation is not essential for its association with the GEM fraction. These results also demonstrate that at least one cysteine at position 3 or 5 is required to generate any binding to the GEM fraction at all and that the apparently free cysteine at position 3 is most critical. The lack of association of G2A with the nonionic detergent-resistant fraction is expected since

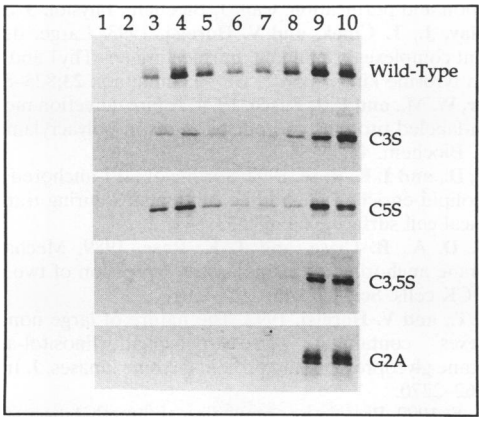

FIG. 8. Association of the p56^{lck} cysteine mutants with the GEM fraction. Wild-type p56^{lck} and each of the p56^{lck} cysteine mutants were expressed in HeLa cells. The cells were labeled with [^{35}S]methionine, chased for 2 h, and lysed with buffer containing 1% TX100. Following equilibrium centrifugation of the lysate, the gradient fractions were immunoprecipitated with rabbit antiserum to p56^{lck}. Fraction 1 represents the top of the gradient. Fractions 3 to 5 were used to represent the amount of protein in the GEM fraction.

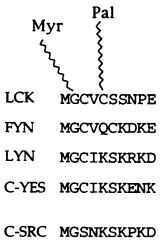

FIG. 9. N-terminal sequences of tyrosine kinases that associate with GPI-anchored proteins. Sequences of the N-terminal 10 amino acids of murine p56^{lck} (52), p56^{lyn} (54), c-YES (20), and pp60$^{c\text{-}src}$ (27) and human p59^{fyn} (19) are shown. p56^{lck}, p59^{fyn}, and p56^{lyn} associate with GPI-anchored proteins, and c-YES associates with the GEM fraction in HeLa cells.

this mutation removes all membrane binding of p56^{lck}. From the data in Fig. 8, we conclude that the myristoylated N terminus and the cysteine at position 3 are critical components of the signal mediating binding of p56^{lck} to the GEM fraction. In the absence of Cys-3, the palmitoylated Cys-5 promotes only a low level of association with this fraction.

Because the C3,5S and G2A mutants did not associate with the GEM fraction, they would not be expected to coimmunoprecipitate with PLAP. To test this, PLAP and either C3,5S or G2A were coexpressed and the GEM fraction was isolated. In each case, only PLAP was associated with the GEM fraction while the p56^{lck} mutants remained entirely TX100 soluble, and there was no coimmunoprecipitation of PLAP and the p56^{lck} mutants C3,5S and G2A (data not shown).

Finally, to determine if any of the p56^{lck} mutants had lost the ability to bind to CD4, wild-type p56^{lck} and each of the mutants were coexpressed with GCD4. GCD4 contains the VSV G ectodomain and the CD4 cytoplasmic and transmembrane domains and has been shown to bind to wild-type p56^{lck} (43). Each of the cysteine mutants bound to GCD4 as effectively as wild-type p56^{lck}, demonstrating that the N-terminal cysteines do not influence this interaction (data not shown).

DISCUSSION

GPI-anchored proteins are located in the outer leaflet of the plasma membrane, while the nonreceptor tyrosine kinases are anchored to the cytoplasmic face of the membrane. The important finding that certain nonreceptor tyrosine kinases are associated with GPI-anchored proteins (12, 45, 46, 48, 49) has raised a major question about the nature of this association. Association might occur through transmembrane proteins such as caveolin that have been found in membrane complexes including GPI-anchored proteins and certain tyrosine kinases (23, 41).

We have expressed GPI-anchored proteins and the nonreceptor protein tyrosine kinase p56^{lck} in HeLa cells and report here that these proteins associated independently with the same low-density GEM fraction that has been characterized previously with MDCK cells (5). Immunoprecipitation of the GPI-anchored proteins or the kinase apparently resulted in precipitation of the entire membrane fraction including the associated proteins. Very gentle disruption of these membranes by warming to 37°C in the presence of TX100 dissociated the interaction of the p56^{lck} with GPI-anchored proteins in the same fraction. These results suggest that the observed interaction of the two proteins arises from their mutual association with the GEM fraction and that binding to a common transmembrane protein intermediate would not be required to explain their interaction.

We have also examined the signals that are involved in targeting GPI-anchored proteins and a tyrosine kinase to the GEM fraction. Results with chimeric proteins demonstrate that the GPI anchor constitutes a signal that is sufficient for targeting proteins to this fraction. An earlier study had shown that the signal determining association of p56^{lck} with a GPI-anchored protein resided in the N-terminal 10 amino acids (46). On the basis of the results reported here it is likely that this signal was actually targeting p56^{lck} to the GEM fraction. It was also suggested in that study that palmitoylation might be a critical part of the signal for association, because mutation of cysteines at positions 3 and 5 abolished palmitoylation as well as interaction with the GPI-anchored protein.

We performed a more detailed analysis of the palmitoylation of p56^{lck} and reached a different conclusion about the nature and role of this modification in p56^{lck}. We found that Cys-5 is nearly quantitatively palmitoylated while Cys-3 is not palmitoylated. Interestingly, the nonpalmitoylated cysteine is the most critical residue for binding to the GEM fraction. In addition, the ability of mutants to bind the GEM fraction correlated directly with their ability to associate with GPI-anchored proteins. In agreement with the idea that the nonpalmitoylated Cys-3 is a critical part of the signal, the four tyrosine kinases known to associate either with GPI-anchored proteins (12, 45, 46, 48, 49) or with the GEM fraction (41) have only this Cys residue and the N-terminal myristylated glycine in common in the first 10 residues (Fig. 9). pp60$^{c\text{-}src}$ does not associate with GPI-anchored proteins and lacks the cysteine at position 3 (12). Thus, the common signal for association of these proteins with the GEM fraction is likely to consist of the myristylated glycine at the N terminus followed by a nonpalmitoylated cysteine.

In addition to p56^{lck}, some α subunits of heterotrimeric G proteins are both myristoylated and palmitoylated (28). In these proteins, N-terminal palmitoylation is limited to the cysteine residue at position 3, the residue that is not palmitoylated in p56^{lck}. Although palmitoylation of α_s appears to be modulated by activation of β-adrenergic receptors, the effect of

this modification on membrane binding properties appears to vary among different α-subunit species. α-subunit palmitoylation may function in interactions with the β and γ subunits of G proteins rather than in direct association with the membrane. Alternatively, α-subunit palmitoylation may have a largely accessory role as shown here with $p56^{lck}$.

Concentration of the GPI-anchored proteins in the GEM fraction may result from direct interaction with glycolipids that are highly enriched in this membrane domain, but a role for other proteins in the domain cannot be ruled out. On the cytoplasmic side of the membrane, the N-terminal myristyl group on $p56^{lck}$ is likely to interact directly with membrane lipids, but the means by which the Cys-3 confers specificity for the GEM fraction is unclear. Enrichment of certain lipids in the inner leaflet may be sufficient to direct binding of $p56^{lck}$. Alternatively, a transmembrane protein such as caveolin that is enriched in this fraction (41) may also be involved in binding. The interactions among many of the protein components in the GEM fraction may also be stabilized by lipids. Communication between the two leaflets could occur either by coupling of the lipids in each half of the bilayer in the GEM fraction or through a transmembrane protein intermediate. Examples of leaflet coupling by lipids exist in some model systems (17), but their applicability to cell membranes remains to be shown.

Interactions between proteins and specific lipids are well documented (11, 26, 29, 34) and are probably important for formation of lipid domains in biological membranes (33). The specific enrichment of sphingolipids, including glycosphingolipids, in the GEM fraction suggests that there are features within these molecules that are responsible for domain formation. Evidence of glycosphingolipid domains in model systems is well documented (reviewed in reference 50). Furthermore, interactions between glycosphingolipids and cholesterol (47) and the presence of phosphatidylinositol domains in biological membranes (16) have been demonstrated and further suggest a mechanism in which lipids are important for formation and stabilization of the GEM fraction.

Coassociation of GPI-anchored proteins and tyrosine kinases with the GEM fraction may serve as a mechanism for assembling signal transduction complexes in the plasma membrane, resulting in the observed activation of T cells by cross-linking of surface GPI-anchored proteins (10, 24, 25). Other functions that may be assigned to the GEM fraction include sorting of GPI-anchored proteins to the apical membrane in epithelial cells (6) and sorting of proteins destined to reside in caveolae (41). The observation that CD4 endocytosis is inhibited by binding to $p56^{lck}$ (31) might be explained by association of the $p56^{lck}$-CD4 complex with the GEM fraction rather than with coated pits involved in endocytosis. The role of the GEM fraction in these various membrane events remains a topic of further investigation.

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REFERENCES

1. **Berger, J., R. Micanovic, R. J. Greenspan, and S. Udenfriend.** 1989. Conversion of placental alkaline phosphatase from a phosphatidylinositol-glycan-anchored protein to an integral transmembrane protein. Proc. Natl. Acad. Sci. USA **86:**1457–1460.
2. **Bligh, E. G., and W. J. Dyer.** 1957. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
3. **Bohuslav, J., T. Cinek, and V. Horejsi.** 1993. Large, detergent-resistant complexes containing murine antigens Thy1 and Ly6 and protein tyrosine kinase $p56^{lck}$. Eur. J. Immunol. **23:**825–831.
4. **Bonner, W. M., and P. J. Fusco.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46:**83–88.
5. **Brown, D., and J. K. Rose.** 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell **68:**533–544.
6. **Brown, D. A., B. Crise, and J. K. Rose.** 1989. Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells. Science **245:**1499–1501.
7. **Cinek, T., and V. Horejsi.** 1992. The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J. Immunol. **149:**2262–2270.
8. **Clarke, S.** 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Annu. Rev. Biochem. **61:**355–386.
9. **Crise, B., A. Ruusala, P. Zagouras, A. Shaw, and J. K. Rose.** 1989. Oligomerization of glycolipid-anchored and soluble forms of the vesicular stomatitis virus glycoprotein. J. Virol. **63:**5328–5333.
10. **Davis, L. S., S. S. Patel, J. P. Atkinson, and P. E. Lipsky.** 1988. Decay-accelerating factor functions as a signal transducing molecule for human T cells. J. Immunol. **141:**2246–2252.
11. **Devaux, P. F., and M. Seigneuret.** 1985. Specificity of lipid-protein interactions as determined by spectroscopic techniques. Biochim. Biophys. Acta **822:**63–125.
12. **Draberova, L., and P. Draber.** 1993. Thy-1 glycoprotein and src-like protein-tyrosine kinase $p53/p56^{lyn}$ are associated in large detergent-resistant complexes in rat basophilic leukemia cells. Proc. Natl. Acad. Sci. USA **90:**3611–3615.
13. **Englund, P. T.** 1993. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Annu. Rev. Biochem. **62:** 121–138.
14. **Fuerst, T. R., E. G. Niles, F. W. Studier, and M. B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
15. **Garcia, M., C. Mirre, A. Quaroni, H. Reggio, and A. Le Bivic.** 1993. GPI-anchored proteins associate to form microdomains during their intracellular transport in Caco-2 cells. J. Cell Sci. **104:**1281–1290.
16. **Gascard, P., M. Sauvage, J.-C. Sulpice, and F. Giraud.** 1993. Characterization of structural and functional phosphoinositide domains in human erythrocyte membranes. Biochemistry **32:**5941–5948.
17. **Haverstick, D. M., and M. Glaser.** 1988. Visualization of domain formation in the inner and outer leaflets of a phospholipid bilayer. J. Cell Biol. **106:**1885–1892.
18. **James, G., and E. N. Olson.** 1990. Fatty acylated proteins as components of intracellular signaling pathways. Biochemistry **29:** 2623–2634.
19. **Kawakami, T., C. Y. Pennington, and K. C. Robbins.** 1986. Isolation and oncogenic potential of a novel human *src*-like gene. Mol. Cell. Biol. **6:**4195–4201.
20. **Klages, S., D. Adam, E. Eiseman, J. Fargnoli, J. M. Dymecki, S. V. Disedrio, and J. B. Bolen.** 1993. Molecular cloning and analysis of cDNA encoding the murine c-yes tyrosine protein kinase. Oncogene **8:**713–719.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lisanti, M. P., I. W. Caras, M. A. Davitz, and E. Rodriguez-Boulan.** 1989. A glycophospholipid anchor membrane anchor acts as an apical targeting signal in polarized epithelial cells. J. Cell Biol. **109:**2145–2156.
23. **Lisanti, M. P., Z. Tang, and M. Sargiacomo.** 1993. Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae. J. Cell Biol. **123:**595–604.
24. **Maino, V. C., M. A. Norcross, M. S. Perkins, and R. T. Smith.** 1981. Mechanism of Thy-1 mediated T cell activation: role of Fc

receptors, T200, Ia, and H-2 glycoproteins in accessory cell function. J. Immunol. **126**:1829–1835.
25. **Malek, T. R., G. Ortega, C. Chan, R. A. Kroczek, and E. M. Shevach.** 1986. Role of Ly-6 in lymphocyte activation. Induction of T cell activation by monoclonal anti-LY-6 antibodies. J. Exp. Med. **164**:709–722.
26. **Marsh, D.** 1990. Lipid-protein interactions in membranes. FEBS Lett. **268**:371–375.
27. **Martinez, R., B. Mathey-Prevot, A. Bernards, and D. Baltimore.** 1987. Neuronal pp60c-src contains a six-amino acid insertion relative to its non-neuronal counterpart. Science **237**:411–415.
28. **Mumby, S. M., C. Kleuss, and A. G. Gilman.** 1994. Receptor regulation of G-protein palmitoylation. Proc. Natl. Acad. Sci. USA **91**:2800–2804.
29. **Narayanaswami, V., J. Kim, and M. G. McNamee.** 1993. Protein-lipid interactions and Torpedo californica nicotinic acetylcholine receptor function. Biochemistry **32**:12413–12419.
30. **Paige, L. A., M. J. S. Nadley, M. L. Harrison, J. M. Cassady, and R. L. Geahlen.** 1993. Reversible palmitoylation of the protein-tyrosine kinase $p56^{lck}$. J. Biol. Chem. **268**:8669–8674.
31. **Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh.** 1992. The protein tyrosine kinase $p56^{lck}$ inhibits endocytosis by preventing entry of CD4 into coated pits. J. Cell Biol. **117**:279–290.
32. **Powell, S. P., B. A. Cunningham, G. M. Edelman, and E. Rodriguez-Boulan.** 1991. Targeting of transmembrane and GPI-anchored forms of N-CAM to opposite domains of a polarized epithelial cell. Nature (London) **353**:76–77.
33. **Rodgers, W., and M. Glaser.** 1991. Characterization of lipid domains in erythrocyte membranes. Proc. Natl. Acad. Sci. USA **88**:1364–1368.
34. **Rodgers, W., and M. Glaser.** 1993. Distributions of proteins and lipids in the erythrocyte membrane. Biochemistry **32**:12591–12598.
35. **Rose, J. K., G. Adams, and C. Gallione.** 1984. The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition. Proc. Natl. Acad. Sci. USA **81**:2050–2054.
36. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
37. **Rose, J. K., L. Buonocore, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10**:520–525.
38. **Rothberg, K. G., J. E. Heuser, W. C. Donzell, Y.-S. Ying, J. R. Glenney, and R. G. W. Anderson.** 1992. Caveolin, a protein component of caveolae membrane coats. Cell **68**:673–682.
39. **Saiki, R. K., S. J. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
41. **Sargiacomo, M., M. Sudol, Z. Tang, and M. P. Lisanti.** 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J. Cell Biol. **122**:789–807.
42. **Schmidt, M. F. G., and M. J. Schlesinger.** 1979. Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein. Cell **17**:813–819.
43. **Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose.** 1989. The *lck* tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. Cell **59**:627–636.
44. **Shaw, A. S., J. Chalupny, J. A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose.** 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. Mol. Cell. Biol. **10**:1853–1862.
45. **Shenoy-Scaria, A. M., J. Kwong, T. Fujita, M. W. Olszowy, A. S. Shaw, and D. M. Lublin.** 1992. Signal transduction through decay-accelerating factor. Interaction of glycosyl-phosphatidylinositol anchor and protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. J. Immunol. **149**:3535–3541.
46. **Shenoy-Scaria, A. M., L. K. Timson Gauen, J. Kwong, A. S. Shaw, and D. M. Lublin.** 1993. Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. Mol. Cell. Biol. **13**:6385–6392.
47. **Slotte, J. P., A.-L. Ostman, E. R. Kumar, and R. Bittman.** 1993. Cholesterol interacts with lactosyl and maltosyl cerebrosides but not with glucosyl or galactosyl cerebrosides in mixed monolayers. Biochemistry **32**:7886–7892.
48. **Stefanova, I., V. Horejsi, I. J. Ansotegui, W. Knapp, and H. Stockinger.** 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science **254**:1016–1019.
49. **Thomas, P. M., and L. E. Samelson.** 1992. The glycophosphatidylinositol anchored Thy1 molecule interacts with the $p60^{fyn}$ protein tyrosine kinase. J. Biol. Chem. **267**:12317–12322.
50. **Thompson, T. E., and T. W. Tillack.** 1985. Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. Annu. Rev. Biophys. Chem. **1985**:361–386.
51. **Vioque, E.** 1984. Spray reagents for thin-layer chromatography (TLC) and paper chromatography (PC), p. 309–316. *In* H. K. Mangold, G. Zeig, and J. Sherma (ed.), Handbook of chromatography, vol. 2. CRC Press, Boca Raton, Fla.
52. **Voronova, A. F., and B. M. Sefton.** 1986. Expression of a new tyrosine protein kinase is stimulated by retrovirus promoter insertion. Nature (London) **319**:682–685.
53. **Whitt, M., and J. K. Rose.** 1991. Fatty acid acylation is not required for membrane fusion activity or glycoprotein assembly into VSV virions. Virology **185**:875–878.
54. **Yi, T., J. B. Bolen, and J. N. Ihle.** 1991. Hematopoietic cells express two forms of *lyn* kinase differing by 21 amino acids in the amino terminus. Mol. Cell. Biol. **11**:2391–2398.
55. **Zurzolo, C., M. P. Lisanti, I. W. Caras, L. Nitsch, and E. Rodriguez-Boulan.** 1993. Glycophosphatidylinositol-anchored proteins are preferentially targeted to the basolateral surface in Fischer rat thyroid epithelial cells. J. Cell Biol. **121**:1031–1039.