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Src-family nonreceptor protein tyrosine kinases (NRPTK) are associated with cell surface receptors in large detergent-resistant complexes: in epithelial cells, yes is selectively located in vesicle structures containing caveolin ("caveolae"). These formations are typically also endowed with glycoprophosphatidylinositol (GPI)-anchored proteins. In the present study, we observed lck, lyn, src, hck, CD4, CD45, G proteins, and CD55 (decay-accelerating factor) expression in the buoyant low-density Triton-insoluble (LDTI) fraction of selected leukemic cell lines and granulocytes. We provide a detailed analysis of the two most highly expressed NRPTK, p53/p56Ick and p56lyn, which are involved in the transduction of signals for proliferation and differentiation of monococytes/B lymphocytes and T lymphocytes, respectively. We show that lyn is selectively recovered in LDTI complexes isolated from human leukemic cell lines (promyelocytic [HL-60], erythroid [K562] and B-lymphoid [697]) and from normal human granulocytes, and that lck is recovered from LDTI fractions of leukemic T- and B-lymphoid cell lines (CEM, 697). In LDTI fractions of leukemic cells, lck and lyn are enriched 100-fold as compared with the total cell lysates. Analysis of these fractions by electron microscopy shows the presence of 70- to 200-nm vesicles: lyn and lck are homogenously distributed in the vesicles, as revealed by an immunogold labeling procedure. These novel results propose a role for these vesicles in signal transduction mechanisms of normal and neoplastic hematopoietic cells. In support of this hypothesis, we further observed that molecules participating in B- and T-cell receptor activation cofractionate in the LDTI fractions, CD45/lyn (B cells) and CD45/lck/CD4 (T cells).

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lymphocytes by immunofluorescence and confocal microscopy.

We report here a preferential localization in plasma membrane low-density Triton-insoluble (LDTI) subfractions of (1) Src kinase proteins: lyn in human leukemic (B-lymphoid, promyelocytic, and erythroid) cell lines and normal granulocytes, and lck in T/B-lymphoid cell lines; (2) GPI-linked protein: CD55 in erythroid, T-lymphoid, and promyelocytic cell lines; (3) transmembrane protein: CD4 in the CEM cell line and CD45 in T lymphoid and normal and leukemic B cells; and (4) α-subunit of the heterotrimERIC G protein in CEM, HL-60, and granulocytes. Finally, morphologic analysis of LDTI fractions showed the presence of 70- to 200-nm vesicles, apparently reminiscent of caveolae. Thus, we provide here for the first time in the hematopoietic system a simple method to isolate a domain of the plasma membrane involved in signal transduction of T and B cells.

MATERIALS AND METHODS

Hematopoietic cell culture. HL-60, CEM, 697, DAUDI, K562, and normal human granulocytes were grown in tissue culture flasks in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Granulocytes were obtained from human adult peripheral blood; the blood was diluted with an equal volume of phosphate-buffered saline (PBS) and underlaid with Ficoll-Paque (Sigma Chemical Co, St Louis, MO) for centrifugation. Granulocytes were harvested from the interface and separated from red blood cells in dextran 3% and 0.15 mol/L NaCl at 37°C for 30 minutes. Residual erythrocytes were lysed with buffer containing 0.15 mol/L NH4Cl, 0.01 mol/L KHCO3, and 0.1 mmol/L EDTA for 10 minutes at 4°C. Flow cytometry analysis showed a 90% granulocyte population.

Antibodies and cell surface markers. Sulfo-NHS-biotin was obtained from Pierce (Rockford, IL). 125I-Streptavidin was purchased from Amersham Life Science (Arlington, IL). Antibodies used were as follows: for immunoblotting, mouse monoclonal anti-CD45 IgG, and anticaveolin rabbit polyclonal antibody were purchased from Transduction Laboratories (Lexington, KY); rabbit polyclonal antilyn, anti-lck, anti-c-src, anti-hck, anti-c-yes, anti-fyn, anti-zap70, and anti-c-fgr IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-CD55 (DAF, Brio 110) was purchased from Upstate Biotechnology Inc (UBI, Lake Placid, NY); mouse monoclonal anti-CD4 was kindly provided by Dr F. Malavasi (University of Torino, Italy); and rabbit anti-Ga serum was a gift from Dr T. Costa (Istituto Superiore di Sanità, Rome, Italy). For immunogold labeling, anti-lyn polyclonal antibody was from Upstate Biotechnology Inc, and anti-lck polyclonal antibody (CT) from Santa Cruz Biotechnology. Na,K-ATPase (α subunit) monoclonal antibody was generously provided by Dr M. Caplan (Yale University).

Isolation of LDTI. LDTI complexes were isolated as previously described. Briefly, 5 × 107 cells were washed and resuspended in 2 mL Mes-buffered saline (MBS) 25 mmol/L, Mes, pH 6.5, and 0.15 mol/L NaCl containing 1% Triton X-100 and 1 mmol/L phenylmethylsulfonyl fluoride and homogenized with 10 strokes of a Dounce homogenizer, adjusted to 40% sucrose, and placed at the bottom of an ultracentrifuge tube. A 5% to 30% linear sucrose gradient was then placed above the lysate, and the mixture was then centrifuged at 39,000 rpm for 16 hours at 4°C in a SW41 rotor (Beckman Instruments, Palo Alto, CA). The visible band migrating as Triton X-100 and 1 mmol/L phenylmethylsulfonyl fluoride and homogenized with 10 strokes of a Dounce homogenizer, adjusted to 40% sucrose, and placed at the bottom of an ultracentrifuge tube. A 5% to 30% linear sucrose gradient was then placed above the lysate, and the mixture was then centrifuged at 39,000 rpm for 16 hours at 4°C in a SW41 rotor (Beckman Instruments, Palo Alto, CA). The visible band migrating at approximately 20% sucrose was harvested and washed twice with MBS at 14,000 rpm for 30 minutes at 4°C, 5 × 107 cells, representing 20% to 22% mg protein, yielded 6 to 10 mg LDTI complexes, ie, approximately 0.02% of the original homogenate.

Detection of surface proteins. Cells were washed three times with PBS and then incubated with sulfo-NHS-biotin (0.5 mg/mL) for 30 minutes at 4°C. After washing with ice-cold serum-free DME medium and then with PBS, cells were homogenized to obtain the LDTI fraction (see above). Proteins from this fraction (20 μg) were then separated on 10% sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. The filter was then incubated with buffer containing 1 mol/L glucose, 10% (vol/vol) glycerol, 0.5% (vol/vol) Tween-20, 3% bovine serum albumin (BSA), and 1% nonfat dry milk (Carnation, Glendale, CA) for 60 minutes at 25°C. After four washes with water, [125I]-streptavidin (1 to 2 × 108 counts/mL) was added to the buffer for 60 minutes at 25°C. Biotinylated proteins were visualized by autoradiography on Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

Kinase assay. LDTI complexes (15 μg) isolated from HL-60, CEM, 697, and K562 were resuspended in 20 μL kinase reaction buffer (20 mmol/L HEPES, pH 7.4, 5 mmol/L MgCl2, and 1 mmol/L MnCl2) and incubated with 10 μCi (γ-32P)ATP for 10 minutes at room temperature. The reaction was stopped by addition of 20 μL Laemmli sample buffer (2×), and the mixture was boiled, separated on 10% SDS-PAGE under reducing conditions, and transferred to nitrocellulose paper.

The bands were visualized by exposure to Kodak XAR film.

Immunoblotting. Sucrose gradients were separated into 13 1-mL fractions. One hundred microliters from each fraction were mixed with 2× Laemmli sample buffer, boiled, and separated on 10% SDS-PAGE under reducing conditions. The insoluble pellet (fraction 13) was solubilized using 10% SDS, heating, dilution with sample buffer, and boiling. In other experiments, total cell lysates and LDTI complexes were first normalized for protein content (Bradford assay) and then analyzed for lyn or lck expression by SDS-PAGE under reducing conditions, followed by transfer to 0.22 μm nitrocellulose paper (Amersham Life Sciences, Buckinghamshire, UK). Blots were blocked with 10 mmol/L Tris, pH 8.0, 0.15 mol/L NaCl, 0.05% Tween-20, and Carnation 1% nonfat dry milk (TBST) for 45 minutes at 25°C. After repeated washes the filter was incubated with 1 μg/mL anti-lck, anti-lyn rabbit polyclonal antibody in TBST for 45 minutes at 25°C or anti-CD45 (0.2 μg/mL), -CD4, -CD55 mouse IgG or anti-Ga rabbit serum dilution 1:2,000 under the same conditions. Bound antibodies were then visualized with a polyclonal or monoclonal horseradish peroxidase (HRP) conjugated secondary antibody diluted 1:5,000, followed by incubation with the enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham Life Sciences) according to the manufacturer’s instructions.

The same conditions were used for the other analyzed src-NRPTK.

Caveolin was detected as follows: LDTI complexes and total cell homogenates were protein-quantified after acetone precipitation. LDTI complexes (5 μg) and total lysate (20 μg) for each cell line were separated on 10% SDS-PAGE and transferred to nitrocellulose. Blots were then incubated with 1 μg/mL anticaveolin polyclonal antibody, raised against the N-terminal cytoplasmic domain of human caveolin (Transduction Laboratories).

Electron microscopy. Triton-insoluble complexes were sedimented at 14,000 rpm for 30 minutes at 4°C. Pellets were fixed in 2% glutaraldehyde in Soerensen’s phosphate buffer for at least 2 hours at 4°C and then postfixed with 1% OsO4 for 1 hour at 4°C and dehydrated in graded ethanol (50% to 100%). Infiltration and embedding were performed in Spurr medium. Blocks were polymerized O/N at 70°C. Thin sections were cut on Ultratcut S (Reichert Jung, Vienna, Austria), stained with uranyl acetate and lead citrate, and examined under the JEOL 100 CX II microscope (JEOL LTDI, Tokyo, Japan).

Immunogold labeling. LDTI complexes were sedimented at 14,000 rpm for 30 minutes at 4°C. Pellets were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol/L PBS, pH 6.5, on ice for 15 minutes. After washing with PBS, samples were embedded.
in 2% agarose, dehydrated in 75% ethanol, and embedded in LR White for 48 hours at 45°C. Thin sections were cut on Ultrcut E and placed on carbon-coated gold grids. Each section was then incubated with 2% BSA and 0.25% Tween-20 in 0.05 mol/L PBS, pH 7.4 (blocker solution) for 30 minutes, followed by incubation with anti-lyn IgG (dilution 1:50) in 1% BSA and 0.05 mol/L PBS, pH 7.4, for 2 hours. The same conditions were used for incubation with anti-lck IgG. After washing with blocker solution, bound antibodies were visualized with a secondary antibody, 5-nm gold-conjugated IgG EM goat antirabbit (Goldmark Biologicals, Phillipsburg, NJ) diluted (1:20) in PBS. The sections were analyzed under the Hitachi H-700 STEM. Control experiments incubated with gold-conjugated secondary antibody indicated that gold labeling was specifically dependent on incubation with anti-lyn and anti-lck IgG. To preserve immunoreactivity with the primary antibodies, it was necessary to omit fixation with Os04, and to decrease the glutaraldehyde level (0.2%), although these conditions are not optimal for structural preservation.

RESULTS

Isolation and characterization of LDTI complexes: Initial analysis. LDTI complexes were isolated from hematopoietic cells according to a recently described procedure, which is based on resistance to solubilization by Triton X-100 at 4°C and buoyancy at a specific density in a linear sucrose gradient. Briefly, HL-60, CEM, 697, and K562 cells were surface-labeled with the membrane-impermeant probe, sulfo-NHS-biotin, to identify LDTI complexes as elements of the plasma membrane. Cells were then homogenized in a buffer at pH 6.5 containing 1% Triton X-100 and adjusted to 40% sucrose. A 5% to 30% linear sucrose gradient was formed above. After centrifugation, each gradient contained a visible opaque band floating at approximately 20% sucrose density. This fraction was separated by SDS-PAGE and blotted with iodinated streptavidin demonstrating cell surface proteins of 100, 97, 72, 50, 32, and 18 kD from LDTI of each cell line (Fig 1A).

A previous study reported that Triton-insoluble fractions isolated from epithelial cells are endowed with kinase activity. We performed a kinase assay on LDTI complexes isolated from HL-60, CEM, 697, and K562. Thus, 15 µg of the LDTI fraction was incubated with γ-32P]ATP and separated by SDS-PAGE. Figure 1B shows the pattern of phosphorylated proteins for each cell line.

In an attempt to identify the phosphorylated proteins, we analyzed src-family NRPTK expression in LDTI complexes from HL-60, K562, 697, and CEM leukemic cell lines and normal granulocytes isolated from peripheral blood. A previous study indicated the presence of lyn at the granulocyte cell surface membrane and c-yes in a lysate of polymorphonuclear leukocytes. Thus, granulocytes were homogenized and sucrose gradient–centrifuged, resulting in an opaque band floating at 20% sucrose density. LDTI complexes (10

![Image](https://example.com/image1.png)

Fig 1. LDTI complexes present surface proteins and kinase activity. (A) HL-60, CEM, 697, and K562 cell lines were surface-biotinylated with sulfo-NHS-biotin, homogenized in buffer containing 1% Triton X-100, and centrifuged in a linear sucrose gradient. 20 µg LDTI complexes were separated on 10% SDS-PAGE and then transferred to nitrocellulose. The filter was probed with [125I]-streptavidin, and biotinylated surface proteins were visualized by autoradiography. (B) Kinase assay. LDTI fraction isolated from HL-60, CEM, 697, and K562 were incubated with [γ-32P]ATP, analyzed on 10% SDS-PAGE, and then transferred onto nitrocellulose. Radioactive proteins were detected by autoradiography. Molecular weights are in kD.
mu g) from HL-60, K562, 697, and CEM cells and normal granulocytes were separated by SDS-PAGE and blotted with 1 mu g/mL src-family polyclonal antibody (Table 1). Positive and negative results obtained for src-family protein expression are shown in Table 1. Particularly, we found src in the LDTI fraction harvested from K562 cells and hck in LDTI complexes isolated from HL-60 cells. In this regard, it has previously been shown that hck is present in the cytosol and membrane fractions of myeloid and B-lymphoid cells.12,13

Interestingly, analysis by immunoblotting of two src-like proteins, zap-70 and syk, produced in both cases negative results (Table 1); this is probably due to their structure diversity from src-family proteins (lack of SH3 domain and myristylation signal).

Lck was observed in the LDTI fraction harvested from 697 and CEM cell lines, although another B-lymphoid cell line (DAUDI) tested by immunoblotting was found negative for lck (Table 1). Lyn was detected in LDTI complexes obtained from HL-60, K562, and 697 cells, and granulocytes.

Focusing on lyn and lck NRPTK expression, we first investigated whether lyn and lck protein expression was restricted to LDTI complexes. After homogenization and sucrose density centrifugation, gradients were fractionated into 13 1-mL fractions. One hundred microliters of each fraction was analyzed by SDS-PAGE and blotted with anti-p56/lck polyclonal antibody. In HL-60 and 697 cells, lyn is predominantly present in 20% sucrose-density fractions 5, 6, and 7 (Fig 2A and B); similar results were obtained in CEM cells, indicating p56/lck protein expression in LDTI complexes in fractions 5, 6, and 7 (data not shown). The LDTI complexes represent 0.02% (4 to 6 mu g) of total proteins (20 to 22 mg) in the initial homogenate (5 x 10^6 cells). Therefore, the LDTI fraction excludes approximately 99% of the total cellular protein that remains with bottom-loaded starting material (the 40% sucrose layer, fractions 8 to 13, Fig 2C).

To exclude the possibility that all plasma membrane proteins partition into the LDTI fraction, we analyzed the expression of an integral plasma membrane protein, Na^+K^-ATPase (alpha subunit) in HL-60 cells (Fig 2D). Na^+K^-ATPase is known to be Triton-insoluble but it fractionates with the high-sucrose-density (30 to 40%) layers.40 After HL-60 homogenization and sucrose-gradient centrifugation, an aliquot from each fraction was acetone-precipitated, separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted. As expected, the largest quantity of this protein partitioned at the bottom layers of the gradient (fractions 8 to 12, Fig 2D), indicating that only a selected part of the plasma membrane segregates as LDTI complexes. This finding is in agreement with our previous study on LDTI complexes isolated from epithelial cells (MDCK), showing that these complexes excluded a battery of organelle-specific marker enzymes for Triton-soluble plasma membrane, Golgi, lysosomes, and ER, which partitioned at the bottom layers of the gradient.40 It has been shown that GPI-anchored proteins partition in detergent-insoluble fractions of the epithelial cell membrane and are enriched in caveolae.25 CD55 (DAF), a GPI-linked protein, has the physiologic role to protect cells from damage by autologous complement and has been detected on the membrane of normal32 and neoplastic19 hematopoietic cells. In line with these studies, we found that CD55 selectively partitions in the LDTI fraction in K562 cells corresponding to the 4 to 6 sucrose gradient fractions (Fig 2E).

LDTI domains are enriched for GPI, Src Proteins, lymphocyte coreceptors, tyrosine phosphatase, and G proteins (alpha subunit). Since lyn and CD55 were mainly expressed in fractions 4 to 7, representing LDTI domains, we investigated the possibility that the colocalization in the LDTI fraction of GPI-linked protein and src kinases indicated the existence of specific domain in the hematopoietic plasma membrane similar to that described in epithelial cells. Thus, we evaluated the fold-enrichment of CD55 in LDTI complexes relative to total cell homogenates in K562, CEM, and HL-60 cell lines (Fig 3A). The results clearly show...
The enrichment of CD55 in the LDTI fraction of each cell line. Granulocytes were also blotted with anti-CD55, resulting in an enrichment of CD55 in the LDTI fraction (data not shown). Coomassie staining (Fig 3B) was used as a control for protein loading. To determine if the different DAF immunoactivities found in the LDTI fractions were related to expression at the cell surface, we performed a CD55 FACS analysis in K562, HL-60, and CEM cells. The FACS data are in agreement with Western blotting results, since in the HL-60 line, DAF-positive cells represent 6% of the population (Fig 3). These findings suggest that cell surface protein density is maintained in LDTI domains.

We then evaluated the enrichment of lyn in LDTI complexes in HL-60 cells. The amount of protein in each fraction was first quantified, and then serially diluted to determine the relative enrichment of lyn (Fig 4A). The enrichment was then quantified by densitometric analysis (not shown). Lyn appeared to be enriched in LDTI complexes up to approximately 100-fold.

To test whether this phenomenon was due to the leukemic lineages used, we analyzed lyn expression in normal human granulocytes isolated from peripheral blood. It has been shown that granulocyte colony-stimulating factor receptor (G-CSFR) signaling involves the formation of a lyn and syk protein complex, indicating the presence of lyn at the granulocyte cytoplasmic surface. Thus, LDTI fractions and total homogenate, derived from granulocytes, were analyzed for lyn expression. Lyn appears approximately 400-fold enriched in the LDTI fraction as compared with the total homogenate (Fig 4B). Normal granulocyte expression of the 56-kD lyn protein is lower than that observed in HL-60 cells using 1 µg protein in both LDTI fractions (Fig 4C). This finding is in agreement with previous results showing higher lyn expression in undifferentiated leukemic cells as compared with normal differentiated cells. Lck was highly enriched (100-fold) within the LDTI fraction in CEM cells (Fig 5A). It has previously been shown that lck is complexed with CD4/CD8 in T cells and CD19/CD21 in B cells (for review, see Weiss and Littman). We investigated if the lck-CD4/CD8 complex is detergent-resistant by immunoblotting CD4 and CD8 expression in the LDTI fraction isolated from CEM cells. CD4 is approximately 10-fold enriched in LDTI complexes (Fig 5B). CD8 was not detectable in CEM cells, but it was found in lymphocytes (data not shown). We further extended our analysis to the CD45 tyrosine phosphatase protein, which is involved in the activation of src kinase catalytic function, and thus in the transduction of signals, through formation of a complex with CD4/lck in T cells and lyn and mlgM in B cells. Thus, LDTI fractions extracted from CEM (T-cell lines), 697 (B-cell line), and peripheral lymphocytes were analyzed for CD45 expression and found to be positive for the antigen (Fig 5C and D). In addition, peripheral lymphocytes and 697 cells presented CD45-lyn colocalization in LDTI complexes (Fig 5D).

Recent studies have been focused on the emerging role of G protein–coupled cell surface receptors (IL-8R) in leukocyte activation. Heterotrimeric G proteins are also enriched in epithelial caveolae. We analyzed G protein expression in hematopoietic vesicles. Results showed an enrichment of Ga subunit in CEM, HL-60, and granulocytes (Fig 5E).

**Morphologic analysis of LDTI-enriched domains.** We examined the morphology of LDTI lyn- and Ick-enriched domains by transmission EM. LDTI domains of each cell...
line appeared as 70- to 200-nm vesicular structures and as membrane fragments that were often curved or U-shaped (Fig 6). Caveolae are smaller than hematopoietic vesicles (50 to 100 nm). A recent study has shown the existence of two vesicle populations in the same system that differ in size and protein content. The hematopoietic vesicles shown here are similar to caveolae in shape, and both are located in the plasma membrane where signal transducers have been found enriched.

In caveolae, variation in the shape of the vesicles might be due to the fact that they can be flat or invaginated and can exist singly or in bunches. Once these structures are no longer linked to the plasma membrane, sectioning of them would be expected to yield fields of closed vesicles and curved membrane fragments. It is likely that this could also contribute to the apparent shape heterogeneity of hematopoietic vesicles.

We next performed immunogold labeling on vesicles isolated from 697, HL-60, and CEM cells (Fig 7). Thin sections of 697 and HL-60 were incubated with anti-lyn IgG (Fig 7A and B), and thin sections of CEM with anti-1ck IgG (Fig 7C). Positive staining indicated that the complexes in these lineages contain lyn and 1ck. To preserve immunoreactivity with antibodies, it was necessary to omit fixation with OsO₄ and to use a small amount of glutaraldehyde (0.2%), al-
fraction and 20 μg total cell homogenates were subjected to immunoblotting with an anticalveolin monoclonal antibody. None of these cell lines showed reactivity with this antibody (Fig 8). Since the antibody used in this assay was raised against a cytoplasmic domain of human caveolin and strongly reacted with the LDTI fraction of MDCK, a canine epithelial cell line used as a positive control, it is likely that the LDTI fraction in hematopoietic cells fails to express caveolin. Indeed, caveolin has been found at high levels in heart, skeletal muscle, placenta, and lung, but is barely detectable in other tissues.46,50

DISCUSSION

We isolated a LDTI plasma membrane fraction enriched with kinase activity from human leukemic cell lines and normal granulocytes. Biochemical results showed the presence of several NRPTK (lck, lyn, src, and hck) in this fraction. We demonstrated that lck or lyn are highly enriched (up to 100-fold) in the LDTI fraction as compared with the total cell lysate in T-lymphoid or promyelocytic cell lines, respectively. In normal granulocytes, lyn was approximately 400-fold enriched in LDTI complexes. Since lck is linked to CD4 in T cells and requires CD45 for activation and coupling to TCR signal transduction,7,9 we investigated the presence of these proteins in LDTI complexes isolated from CEM cells: interestingly, CD4, CD45, and lck colocalized in these complexes. Similarly, CD45 was found in association with lyn in lymphocytes and 697 LDTI domains (see below). Finally, the detergent-insoluble fraction from CEM, HL-60, and granulocytes presents an enrichment for the α subunits of the heterotrimeric G protein. Morphologic analysis demonstrated that these detergent-insoluble complexes isolated from erythroid, promyelocytic, and T- and B-lymphoid cell lines and granulocytes resemble 70- to 200-nm vesicular structures. Immunogold labeling revealed that lck and lyn proteins are localized on LDTI vesicles. The possibility that vesicle formation is an artifact due to the use of detergent during the initial homogenization step has been excluded by our previous study,69 in which caveolae were isolated in absence of ‘‘light vesicles.’’ Using this protocol, we obtained results comparable to those observed including Triton X-100, 50- to 100-nm vesicles enriched in IP3 receptor and caveolin having similar buoyant density and resistance to Triton solubilization.

Detergent-insoluble domains composed of 50- to 100-nm vesicles, known as caveolae, have been isolated from an epithelial cell line.31 As mentioned earlier, several features suggest a relationship between the hematopoietic vesicles isolated and characterized here and the epithelial caveolae: (1) an identical isolation procedure, (2) similar morphology, (3) comparable size (the slightly larger size of hematopoietic vesicles may reflect cell-type–specific variation), (4) GPI-anchored protein (CD55) selective distribution in a detergent-insoluble fraction, and (5) intrinsic kinase activity. In this regard, caveolae show a selective distribution of src kinases (src, yes, lyn, lck, c-fgr, and jak-2)48 and caveolin and c-yes phosphorylation on tyrosine and serine residues.28 Furthermore, the pattern of phosphorylation appears

though these conditions are not optimal for preservation of the original vesicular structure.

Hematopoietic LDTI complexes are caveolin-negative. Caveolin is a principal structural component of caveolae-membrane domains and is detected as two major species migrating at 22 to 24 kD.66,67 We evaluated the expression of caveolin by Western blotting in K562, 697, CEM, and HL-60 cells and granulocyte membranes.* Thus, 5 μg LDTI

* During review of the manuscript, Fra et al (J Biol Chem 269:30745, 1994) showed the lack of caveolin in detergent-insoluble microdomains in lymphocytes and a GPI-anchored protein enrichment (Thy-1) in the same fraction.
Fig 5. LDT1 fraction represents signal transduction complex. (A) p56^<sup>lck</sup> enrichment, (B) CD4 enrichment, and (C) CD45 enrichment in CEM cells. In all cases, proteins from LDT1 fractions and total cell homogenates were quantified, serially diluted (1:10), separated on 10% SDS-PAGE, and transferred to filter. Lck and CD4 expression were detected by blotting with 1 μg/mL anti-Lck or anti-CD4. CD45 protein expression was detected by blotting with 0.2 μg/mL anti-CD45. Enrichment was quantified by densitometric analysis. A commercially available Jurkat cell lysate was used as control for CD45 expression. (D) p53/p56<sup>lck</sup> and CD45 colocalization in lymphocytes and 697 cells. Human peripheral lymphocytes obtained from healthy donors, after previous separation by Ficoll and Percoll gradient were lysed and subjected to sucrose density gradient for LDT1 fraction isolation. After centrifugation, LDT1 complexes recovered were treated as described earlier. (E) Gα enrichment in CEM, granulocytes, and HL-60 cells. Expression of Gα chain was detected by immunoblotting with a rabbit antiserum diluted 1:2,000, followed by the other procedures for immunoblotting. An enrichment of ~10-fold for HL-60 and sixfold for CEM was evaluated by densitometric analysis. The multiple Gα bands detected in the LDT1 fraction in CEM cells may account for several Gα subunits expressed in lymphocytes (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>).

specific to caveolin-rich domains, as suggested by the distinct pattern of phosphorylated proteins when compared with total cell lysate. Hematopoietic vesicles are also endowed with kinase activity, as determined by a selective localization of several NRPTK (lyn, lck, and possibly hck and src) in these domains. Finally, as previously detected in caveolae, we found a selective localization of Gα protein in CEM, HL-60, and granulocyte LDT1 domains. The activation state of these signal transducers is not known, although our recent study indicated that caveolin cofractionates with
inactive Go subunits, suggesting that caveolae may function as inactive signaling-molecule storing sites.

The multiple similarities of caveolae in epithelial cells and caveolae-like vesicles in hematopoietic cells suggest that the signal transduction role postulated for these epithelial structures may also apply to the hematopoietic ones.

Recent studies on the activation of T lymphocytes showed the recruitment of lck and lyn, another src-family related protein, in the cascade signals initiated by TCR activation. Furthermore, lck has been intracellularly localized in interphase T cells at the level of the plasma membrane associated with vesicular structures surrounding the centrosome.

The present results show the preferential distribution of lck and other cytoplasmically oriented protein-tyrosine kinases such as lyn in vesicle-like domains of the plasma membrane of T cells and other hematopoietic cell types. In addition, it has been demonstrated that the recruitment of lck/CD4/CD8 complex is essential for signal transduction at low antigen concentration in T cells, and the protein tyrosine phosphatase CD45 is required for TCR activation in that it dephosphorylates the negative regulatory site of src kinases. Interestingly, LDTI fractions isolated from CEM cells were found to be positive and enriched for lck, CD4, and CD45. Moreover, studies have suggested that CD45, mlgM, and lyn form a complex in B cells, the ability of which to transduce signals may depend on the regulation by tyrosine phosphorylation and dephosphorylation. As expected, CD45 selectively partitions with lyn in LDTI domains from lymphocytes and 697 cells. Altogether, our results on normal and leukemic T and B cells further support the hypothesis that hematopoietic caveolae-like vesicles are functionally involved in signal transduction pathways.

We suggest therefore that our LDTI fraction model offers a tool to study molecular mechanisms underlying hematopoietic cell proliferation and differentiation, particularly for lck and lyn. In this regard, lyn forms a complex with syk, another
cytosolic src-like protein-tyrosine kinase, and G-CSFR in G-CSF–stimulated human neutrophils, indicating the involvement of lyn in the G-CSF signaling pathway. Syk or Zap-70 expression was not detected in myeloid and T-lymphoid cells or in B- and T-lymphoid cell lines, respectively. One possible explanation is that syk and Zap-70 are not myristylated at their domain N-terminals and hence are probably not constitutively localized at the plasma membrane in a cell basal state. Future studies on lyn/G-CSFR association in the LDTI lyn-enriched domain from stimulated neutrophils may help to define the basis for the association of lyn with G-CSFR and the formation of the complex with syk, as well as to identify other signal transducers acting "downstream" of lyn activation.

The lack of caveolin in hematopoietic cells and vesicles does not rule out the existence of caveolae. As recently reported, caveolin moves from the cell surface membrane to the Golgi region without altering caveolae morphology. Thus, it is possible that cells present functional caveolae without caveolin. Another hypothesis is that a different structural protein with the features of epithelial caveolin may be present in hematopoietic vesicles. We performed Coomassie staining on sucrose-gradient fractions from HL-60 cells, indicating the presence of proteins ranging from 22 to 24 kD in LDTI fractions (4 to 6) and bottom layers (8 to 12). It has not been possible to identify a hematopoietic-specific caveolin, although several members of the caveolin gene family are currently being scrutinized. Alternatively, as recently reported by others, detergent-insoluble complexes composed by GPI-anchored proteins, glycosphingolipids,
and src-family proteins are organized in rafts in the cell plasma membrane with or without caveolin.53

In conclusion, we isolated and characterized plasma membrane domains resembling vesicles that assemble GPI-linked proteins (CD55) and multiple molecules involved in the signal transduction machinery (lyn, lck, CD4, CD45, and G proteins).

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Signal transduction and glycophosphatidylinositol-linked proteins (lyn, lck, CD4, CD45, G proteins, and CD55) selectively localize in Triton-insoluble plasma membrane domains of human leukemic cell lines and normal granulocytes

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