

Incorporation of Leucocyte GPI-Anchored Proteins and Protein Tyrosine Kinases into Lipid-Rich Membrane Domains of COS-7 Cells

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Several human leucocyte surface glycoproteins and two lymphoid protein kinases were transiently expressed in monkey COS-7 fibroblastoid cells. All glycosylphosphatidylinositol (GPI)-anchored proteins (CD14, CD16B, CD48, CD59, CD87 and GPI-anchored versions of CD2 and CD25) and protein tyrosine kinase (PTK) Lck but not transmembrane proteins (CD2, CD4, CD5, CD6, CD8) and PTK ZAP-70 were in part localized in buoyant, lipid-rich, detergent-resistant membrane GPI-microdomains of the COS cells. Endogenous GPI-microdomains of COS cells appear to be, in contrast to those present in leucocytes, essentially devoid of associated PTKs. Our results indicate that GPI-anchor is sufficient to target proteins to these membrane specializations even if expressed ectopically. Moreover, the N-terminal double acylation of the PTK Lck appears to be functional also in COS cells and targets the enzyme to the membrane GPI-microdomains implicated in receptor signalling. © 1998 Academic Press

A distinct, structurally diverse group of membrane proteins are those anchored in the lipid membrane via glycosylphosphatidylinositol (GPI) moiety (reviewed in Ref. 1). Among them are e.g. adhesion molecules (CD48, CD58, CD66, Thy-1/CD90), LPS receptor of monocytes CD14, complement-protecting proteins CD55 and CD59, Fc γ -receptor CD16, protease receptor

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Abbreviations: FITC, fluorescein isothiocyanate; GAM, goat F(ab)₂ anti-mouse immuno-globulin; GPI, glycosylphosphatidylinositol; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBL, peripheral blood leucocytes; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PHA, phytohemmagglutinin; PI3-K, phosphatidylinositol 3-kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; PTK, protein tyrosine kinase.

CD87, ectoenzymes (alkaline phosphatase, 5'-nucleotidase (CD73)) and others. A striking feature of these molecules is that they appear to reside mainly in specific areas of the plasma membrane (GPI microdomains; also called sphingolipid-cholesterol membrane domains or glycolipid rafts) enriched in glycosphingolipids, sphingomyelin and cholesterol but relatively poor in phospholipids and transmembrane proteins. These GPI microdomains are associated with protein tyrosine kinases (PTKs) of the Src family and with trimeric G-proteins. The GPI microdomains are resistant to solubilization in detergents such as Triton X-100 or CHAPS; in the presence of such detergents the "GPI complexes" (also called detergent insoluble glycolipid-rich complexes) behave as relatively large particles (around 100 nm) of low buoyant density which can be isolated by density gradient centrifugation (2-9; for review see Ref. 10). It seems likely that these structures are responsible for the well known phenomenon of signal transduction via GPI-anchored proteins and glycolipids and that they may participate also at signalling via T cell receptor and Fc receptors. It is not clear which factors are responsible for maintaining integrity of the GPI microdomains. Namely, it is not clear whether it is only the GPI anchor which targets the GPI-anchored proteins into the microdomains or whether cell-type specific protein-protein or protein-glycolipid interactions play some role. A previous paper indicated that recombinant (chimeric) proteins composed of extracellular part of a normally transmembrane protein or a secreted protein and GPI anchor were not associated with the protein kinase containing complexes upon ectopic expression (11). This could mean that only endogenous GPI-anchored proteins are properly sorted into the GPI microdomains and thus the presence of the GPI anchor may not be sufficient for the incorporation.

The association of Src family kinases with the GPI microdomains is dependent on their N-terminal double-acylation (12, 13); again it is not clear whether this

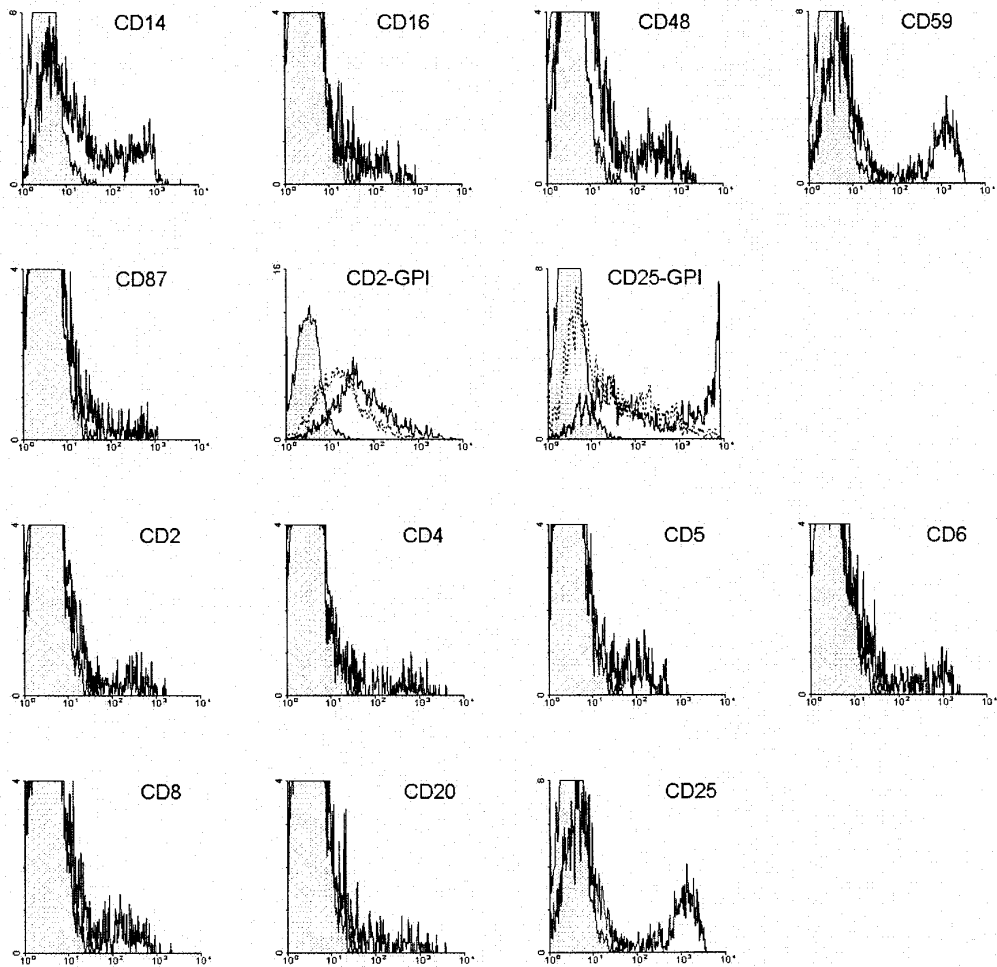


FIG. 1. Expression of the transfected proteins in COS-7 cells as detected by cytofluorometry (standard histograms). Solid lines (shaded peaks): staining with an irrelevant control mAb; solid lines (unshaded peaks): staining with mAbs to the indicated antigens expressed after transfection; dotted lines: staining of the transfectants after PI-PLC treatment (shown only in CD2-GPI and CD25-GPI).

modification is sufficient or whether other cell-type specific factors are important.

In the present study we expressed several "naturally" GPI-anchored proteins, GPI-anchored versions of transmembrane proteins and transmembrane proteins, all of human leucocyte origin, in monkey fibroblastoid COS-7 cells and examined their presence within or outside the GPI microdomains. Similarly, we expressed in these cells human leucocyte-specific protein tyrosine kinases Lck and ZAP-70 and examined whether they will be targeted into the GPI-microdomains.

MATERIAL AND METHODS

Antibodies. MAbs to human proteins CD5 (MEM-32), CD6 (MEM-98), CD14 (MEM-18), CD16 (MEM-154), CD20 (MEM-97), CD48 (MEM-102), CD59 (MEM-43 and MEM-43/5), MHC class I

(MEM-147), Lck (LCK-01) and to phosphotyrosine (P-TYR-01) were previously produced and characterized in our laboratory. MAb TS2/8 (to CD2) was a product of hybridoma obtained from ATCC; Leu3a (to CD4) was from Becton Dickinson (Mountain View, CA); C8 144/B (to CD8; Ref. 14) was kindly provided by Dr. D. Y. Mason; C8.26A3 (to CD87) by Dr. V. H. Weidle; B-F2 (to CD25) by Dr. J. Wijdenes; HB13h (to a denaturation-resistant intracellular epitope of CD20; author T.F. Tedder) was obtained within the B cell panel of 6th International Workshop on Human Leucocyte Differentiation Antigens; Hermes-3 (to CD44) was a gift from Dr. E.C. Butcher; rabbit antiserum to ZAP-70 by Dr. A. C. Chan; rabbit antibody to PI3-K (UBI, Lake Placid, NY) was kindly provided by Dr. B. Schraven; rabbit antibodies to Lyn, and G_{β} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA constructs. The pCDM8-based expression plasmids encoding full-length cDNA of human cell surface molecules CD2, CD4, CD8, CD14, CD16, CD20, and CD5, CD6, CD25, CD87, were kindly provided by Dr. B. Seed and Dr. H. Stockinger, respectively. Expression plasmids pRK5-Lck and pRK5-ZAP70 encoding cDNAs for human tyrosine kinases Lck and ZAP-70 were a gift from Dr. W. Kola-

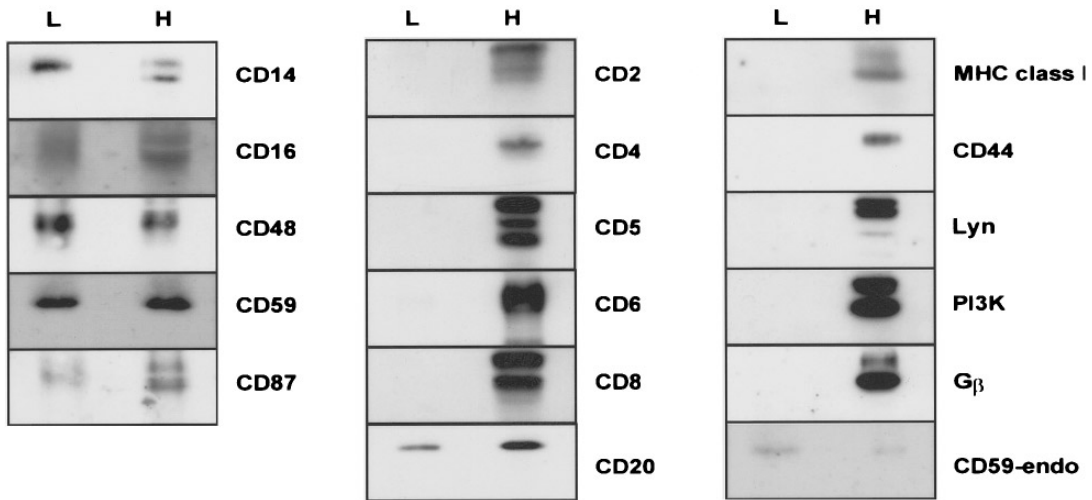


FIG. 2. Incorporation of the protein products of transfected cDNA into the buoyant GPI-complexes in COS-7 cells. Fractions 1 (light, L) and 6 (heavy, H) of the sucrose density gradient were analyzed by SDS PAGE and Western blotting using antibodies to the indicated proteins for immunostaining. The proteins anchored via GPI are shown in the left column; the transmembrane proteins are in the middle column; endogenous membrane and cytoplasmic proteins of COS-7 cells are in the right column. Only the relevant parts of the blots (corresponding to the molecular mass of the respective proteins) are shown.

nus. The cDNAs encoding human CD48 and CD59 were cloned in this laboratory in the pCDM8 vector. Pfu DNA polymerase (Stratagene, La Jolla, CA) with high proofreading rate and the full-length cDNAs of human CD2, CD25 and CD59 were used for the construction of pcDNA-CD2-GPI and pcDNA-CD25-GPI expression plasmids. First, the sequence encoding C-terminal 31 amino acids of CD59 (signal sequence for attachment of the GPI anchor) was amplified using oligonucleotides OLI#018 (**actagcggccgcgaacagcttgaaat-ggtgggaca**) and OLI#19 (**catctagacttagggatgaaggctccaggctgc**) and cloned into *NotI* and *XbaI* sites of pcDNAI/Amp vector (Invitrogen, Carlsbad, CA), resulting in pcDNA-GPI vector. To create pcDNA-CD2-GPI, the sequence encoding entire extracellular part of CD2 including signal peptide was amplified by PCR using oligonucleotides OLI#020 (**caggaagcttaagatgagctttccatgtaa**) and OLI#021 (**actcggcggcctgacaggctcgactgga**), and cloned into *HindIII* and *NotI* sites of the pcDNA-GPI vector. The pcDNA-CD25-GPI vector was constructed in a similar way, but using oligonucleotides OLI#022 (**cagaagcttaagatggattacactgctg**) and OLI#023 (**actcggcggcct-cgtctccatgggtgcacca**) for PCR amplification of the sequence en-

coding the entire extracellular part of CD25. Both constructs were tested for transient expression of the chimaeric proteins by transient transfection in COS-7 cells.

Transfections, detection of the expressed surface proteins and PI-PLC treatment. COS-7 cells were transiently transfected using a modification of the DEAE-dextran method (15); the cells were harvested 48 h after transfection. The percentage of cells expressing the protein products of the transfected cDNA was typically in the range of 10-50%. To detect surface expression by cytofluorometry, cells were incubated 30 min on ice with solutions of mAbs (approx. 10 $\mu\text{g/ml}$) and after washing 30 min with solution of FITC-labeled GAM (10 $\mu\text{g/ml}$). After washing the cells were analyzed on FACSsort cytometer (Becton Dickinson, Mountain View, CA). To test the GPI-anchorage, the cells (10^7) were incubated 1 h in 1 ml of PBS containing PI-PLC (10 μl of the ICN preparation). After washing the cells were labeled with mAbs and analyzed by cytofluorometry.

Density gradient ultracentrifugation and other biochemical methods. 5×10^7 cells were lysed on ice in 1 ml of isotonic lysis solution

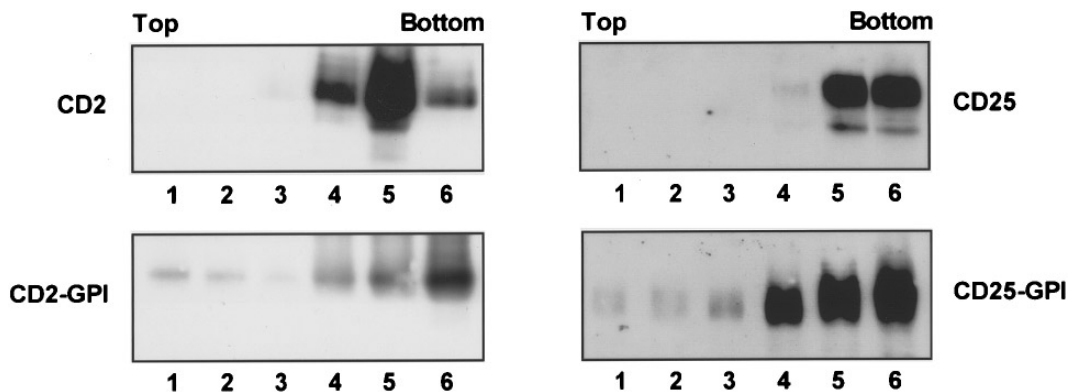


FIG. 3. Incorporation of GPI-anchored (GPI-) and transmembrane versions of CD2 and CD25 proteins into the buoyant GPI-complexes in COS-7 cells. Fractions 1 - 6 of the sucrose gradient density gradient were analyzed by SDS PAGE and Western blotting. Only the relevant parts of the blots (corresponding to the molecular mass of the respective proteins) are shown.

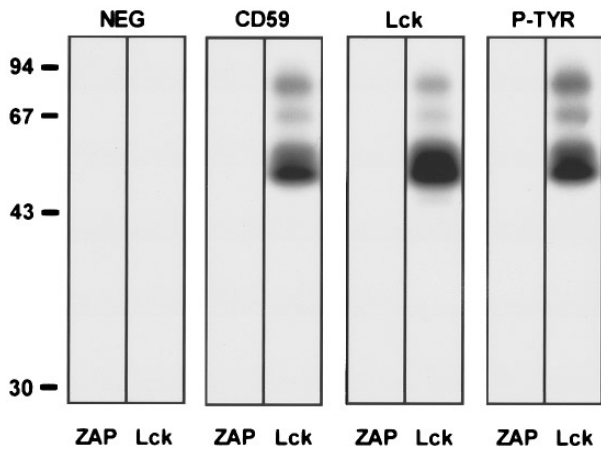


FIG. 4. Results of *in vitro* kinase assay. Detergent lysates of COS-7 cells transfected with human Lck (Lck) or ZAP-70 (ZAP) were immunoprecipitated by mAbs to the molecules indicated at the top (NEG. - negative irrelevant control; CD59, Lck and P-TYR - phosphotyrosine), the immunoprecipitates subjected to *in vitro* kinase assay, SDS PAGE and autoradiography. Immunoprecipitates of the untransfected cells were also negative (not shown). Positions of mol. mass standards (kDa) are shown at the margin.

(Tris-buffered saline containing 1% detergent NP-40 and protease inhibitors 5 mM iodoacetamide, 2mM EDTA and 1 mM Pefabloc). After removal of nuclei by low speed centrifugation (3 min., 3000×g), the supernatant was mixed on ice 1:1 with 80% sucrose in lysis solution. The sample (100 μ l of detergent cell lysate in 40% sucrose) in ultracentrifugation cuvette was overlaid with 500 μ l of 30% sucrose in the lysis solution and with 200 μ l of lysis solution without sucrose. After 20 h centrifugation at 40 000 rpm and 4° C (L7 ultracentrifuge; Beckman) the gradient was fractionated into 6 fractions of identical volume (No. 1 to 6, numbered from top to bottom). The fractions were analysed by SDS PAGE and Western blotting.

Immunoprecipitation (by the solid phase immunoisolation technique using antibody-coated plastic wells), *in vitro* kinase assay (performed on the immunoprecipitates), SDS PAGE and Western blotting (using luminographic detection of the electroblotted proteins) were all performed as described in more detail elsewhere (15).

RESULTS

All recombinant human leucocyte surface proteins including the GPI-anchored versions of CD2 and CD25 were well expressed on the surface of COS-7 cells; as expected, only those anchored via the GPI moiety were largely removed by PI-PLC treatment (Fig. 1 and data not shown). The transfectants were solubilized in a solution of 1% NP-40, and subjected to sucrose density gradient ultracentrifugation. A significant fraction of all GPI-anchored but not transmembrane proteins was present in the low density fractions of the gradient apparently corresponding to the GPI-complexes. The only exception from this rule was the four-transmembrane protein CD20, a fraction of which was also in part found in these light complexes (Fig. 2). The light complexes contained also a fraction of GPI-anchored versions of the natu-

rally transmembrane proteins CD2 and CD25 (Fig. 3). These light complexes contained also a fraction of an endogenous GPI-anchored protein of COS cells, CD59 (Fig. 2) while endogenous CD44, MHC class I as well as PI3-K, Lyn and trimeric G-proteins were not detectable in these complexes (Fig. 2). It should be noted that human CD59 expressed in the transfectants was detected by the same mAb as the endogenous monkey CD59; however, the intensity of staining of the latter was substantially weaker than that of the former, either due to lower level of expression or due to weaker reactivity of the anti-human mAb with the endogenous monkey molecule. Thus, the ectopically expressed leucocyte GPI-anchored proteins were all, in contrast to transmembrane proteins, partially targeted to the detergent resistant, lipid-rich membrane microdomains of the fibroblastoid COS cells.

The negative results of *in vitro* kinase assays on immunoprecipitates obtained by means of mAb to an endogenous GPI-protein of COS cells, CD59, and to phosphotyrosine, indicated that the GPI-microdomains of these cells, in contrast to lymphoid cells, are essentially devoid of associated protein kinases (Fig. 4). In agreement with the result of the *in vitro* kinase test, no endogenous PTK Lyn could be detected in the density gradient fractions corresponding to the GPI-complexes (Fig. 2). However, a strong protein tyrosine kinase activity could be detected in such immunoprecipitates from COS cells transfected with cDNA encoding the Src family kinase Lck (Fig. 4). After separation by sucrose gradient ultracentrifugation, a significant fraction of the recombinant Lck but not of another control recombinant kinase, ZAP-

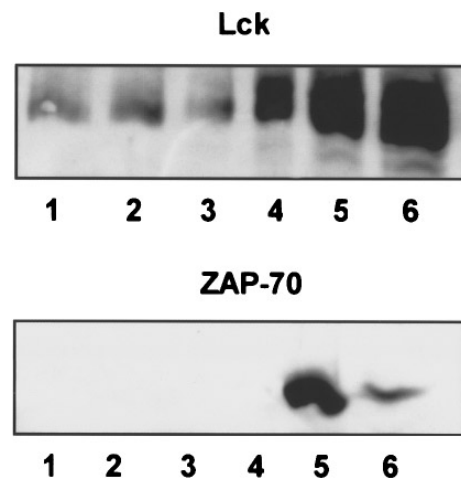


FIG. 5. Distribution of transfected Lck and ZAP-70 in the density gradient fractions. The kinases were detected by Western blotting. Only the relevant parts of the blots (corresponding to mol. mass of the proteins) are shown.

70, was present in the low density detergent-resistant complexes (Fig. 5).

DISCUSSION

The results presented in this paper indicate that the GPI anchor generally targets the proteins carrying it to the detergent-resistant membrane microdomains (the GPI-microdomains) even if expressed ectopically in "unnatural" cells. Such microdomains apparently exist also in the untransfected COS cells and contain endogenous GPI-anchored proteins, such as CD59, which was the only one against which suitable mAb was available (cross-reactive with the monkey molecule). The only leucocyte transmembrane protein expressed in COS cells transfectants which was in part targeted to the GPI microdomains was CD20. This could be due to high hydrophobicity of its four-transmembrane domains; however, this finding is still somewhat surprising as CD20 has not been so far described as a protein component of the GPI domains in B lymphocytes where it is naturally expressed. It may be speculated that this difference reflects some differences in lipid composition of these domains in fibroblasts and B lymphocytes. Thus, our results do not support the earlier indication that GPI anchor by itself may not be sufficient for targeting proteins into the PTK-containing GPI microdomains (11). An explanation for this discrepancy is probably due to the fact that the stable transfectants used by Clissold (11) were cultured in the presence of the neomycin analogue G418 which was recently found to induce release of GPI-anchored proteins in membrane vesicles (16). These vesicles may actually be derived from the PTK-containing GPI-microdomains and the remaining cell surface GPI-anchored proteins may be only those residing outside these microdomains. This possibility is now under study in our laboratory.

The endogenous GPI microdomains in either untransfected COS cells or in those transfected with cDNAs encoding various GPI-anchored leucocyte proteins contained very little if any associated protein kinases detectable by the *in vitro* kinase assay. However, lymphocyte-specific Src family PTK Lck expressed in the COS cells became readily incorporated into the COS cell GPI-microdomains. This indicates that the N-terminal dual acylation of this kinase occurs also in

these non-leucocyte cells and targets the modified enzyme into the GPI-microdomains. In contrast, another PTK, ZAP-70 devoid of the membrane-targeting acylation modification was not (as expected) found in these specific membrane structures. Our results among others indicate that different cell types (leucocytes vs. fibroblasts) may markedly differ in the content of protein kinases in their membrane GPI microdomains. This difference might help to elucidate so far unclear biological role of the GPI microdomains.

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