Detergent-insoluble Glycolipid Microdomains in Lymphocytes in the Absence of Caveolae*

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Antibody binding to glycolipids and glycosphatidyl-

Dylinostitol (GPI)-anchored proteins of lymphocytes can
triger activation of specific signal transduction path-
ways. The finding that GPI-anchored proteins are pre-
SENT in detergent-insoluble complexes with several tyro-
sine kinases of the Src family suggested that these
complexes may represent membrane microdomains in-
volved in the transduction of signals to the cell interior.
Recent work has suggested a link between deterg-
ent-insoluble microdomains and plasma membrane in-
vaginations termed caveolae. Here we show that lym-
phocytes lack plasma membrane domains with the char-
acteristic features of caveolae. Furthermore, VIP21-
caveolin was not detectable in four different lymphocyte
cell lines at the protein or mRNA level. In addition to the
lack of caveolar domains, capping experiments sug-
gested that the bulk of the GPI-anchored protein Thy1
did not associate in the lympocyte plasma membrane. Despite this,
Thy1 and Gαs were present in detergent-insoluble com-
plexes. We conclude that detergent insolubility does not
correlate with the presence of caveola or of VIP21-
caveolin and that caveolae, as defined by a number of
different markers, are not involved in signal transduc-
tion in lymphocytes.

Cross-linking of glycosphatidylinositol (GPI)-anchored
surface glycoproteins can trigger the activation of specific signal
transduction pathways in lymphocytes (for review see Ref.
1). As GPI-anchored proteins are anchored in the outer leaflet
of the lipid bilayer, the mechanism by which such signals are
transmitted across the bilayer to the cytoplasmic surface is not
clear. The finding that GPI-anchored proteins are present in insoluble complexes with tyrosine kinases of the Src family
(2–6) suggested the existence of membrane microdomains that
might be involved in transduction of the transmembrane sig-
als. Based on characterization of detergent-insoluble com-
plexes, these microdomains would consist of GPI-anchored pro-
teins associated with specific glycolipids in large structures of
about 100 nm in diameter (2, 3, 6). The association of GPI-
anchored proteins with these domains is dependent on the GPI
anchor; transmembrane forms of the same proteins do not as-
sociate with Src kinases and cannot transduce signals across
the membrane (5). In addition, association of the Src kinases,
p56lck and p59fyn, with GPI-anchored proteins was shown to
require palmitoylation of their amino termini (7, 8).

Glycolipid-based microdomains may represent a general
phenomenon for segregation of membrane constituents within
the plane of a membrane. In epithelial cells detergent-insoluble
membrane domains were shown to be enriched in newly syn-
thesized GPI-anchored proteins, glycosphingolipids (GSLs),
and cholesterol (9, 10). As GPI-anchored proteins and viral
proteins destined for the apical surface acquire detergent in-
solubility in a late Golgi compartment the results are consist-
ent with the hypothesis that apically destined proteins are
segregated into GSL-enriched microdomains in the trans-Golgi
network of epithelial cells before transport to the cell surface
(11). Detergent insolubility has proven a powerful tool to enrich
for components of membrane microdomains (12, 13). The first
component of these detergent-insoluble complexes to be iso-
lated was VIP21-caveolin (12). VIP21-caveolin was subse-
quently shown to be present in the trans-Golgi network, exo-
cytic vesicles, and in the plasma membrane invaginations
termed caveolae (14). Caveolae are enriched in certain GSLs
(15) and in GPI-anchored proteins (16) and therefore show
some of the characteristics of glycolipid microdomains. Subse-
quently, detergent-insoluble material isolated from MDCK cells
was shown to contain a SR-like kinase and tyrosine kinase
substrates (13). It was therefore suggested that caveolae, and
in particular VIP21-caveolin, are involved in signal transduc-
tion. In this paper we investigated the possible role of caveolae
in lymphocytes in which the signal transduction machinery
and pathways are well characterized.

MATERIALS AND METHODS

Cell Lines and Antibodies—A20 (IgGk BALB/c mouse B lymphoma
(17)), HeLa, and NIH-3T3 cell were cultured in DMEM, 10% fetal calf
serum; A20 medium was supplemented with 50 µg 2-mercaptoethanol.
P3X63-Ag8.653 (Ag8) (Ig mouse myeloma), Jurkat (human T lym-
phoma), and 2B2318 (mouse T hybridoma (18)) were maintained in
RPMI, 10% fetal calf serum. MDCK strain II and A431 cells were
cultured as described previously (14). The monoclonal anti-caveolin
antibody was obtained from Transduction Laboratories (Lexington,
KY). The affinity-purified rabbit anti-VIP21 NH2-terminal peptide
(19, 20) was used as described previously (14). The rabbit
anti-Thy1 serum and the rabbit anti-mouse integrin β1 serum were
kindly provided by Claude Bron (Institute of Biochemistry, University
of Lausanne, Switzerland) and by Paolo Bernardi (Department of Bio-
medical Sciences, University of Padova, Italy), respectively.

Western Blot—Western blot analysis was performed as previously
described (14). Thy1 and VIP21-caveolin were analyzed by 15% SDS-
polyacrylamide gels (19) under reducing conditions, while nonreducing
8% gels were used for integrin pl.

Northern Blot—Total RNA was prepared using the single-step acid
guanidinium thiocyanate/phenol/chloroform extraction (20). RNA
samples (10 µg) were separated on a 1% agarose, 6% formaldehyde
gel and transferred in 10 x SSC to a Hybond-N membrane (Amersham
Corp.). The membrane was prehybridized for 2 h at 42 °C in 5 x SSC,
50% formamide, 5 x Denhardt’s solution, 1% SDS (21) and then
incubated overnight with the 32P-labeled probe dissolved in the same so-

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‡The abbreviations used are: GPI, glycosylphosphatidylinositol; CT,
cholera toxin binding subunit; MDCK, Madin-Darby canine kidney
cells; GSL, glycosphingolipid; DMEM, Dulbecco’s modified Eagle’s me-
dium; PBS, phosphate-buffered saline; BSA, bovine serum albumin;
HBSS, Hank’s buffered salt solution; HRP, horseradish peroxidase; Gαs,
monosialoganglioside.
lution. After hybridization the membrane was washed with 0.1 x SSC, 1% SDS and exposed to x-ray films. Probes were prepared by random priming (Amersham Corp.) from MDCK VIP21 cDNA (12) and from a 283-base pair poly(A) chain reaction fragment derived from human glyceraldehyde phosphate dehydrogenase cDNA.

**Detergent Extractions**—The detergent extraction and flotation protocols were adapted from previously described procedures (9, 10). 2 x 10^7 2B2318 cells, preincubated for 30 min with 5 µg/ml CT-HRP in DMEM, 10 µM Hepes, 0.2% BSA were washed once in PBS and extracted for 30 min on ice with 500 µl of TNE/Triton X-100 (25 µM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Trition X-100, and a mixture of protease inhibitors containing 30 µg/ml each of chymostatin, leupeptin, antipain, and pepstatin). The extract was brought to 1.5 µs acrose in a SW 40 centrifuge tube, overlaid with 8.5 ml of 1.2 and 2.5 ml of 0.15 µs acrose in 10 µs Tris-HCl, pH 7.5, and centrifuged for 18 h at 38,000 rpm at 4 °C. 1-ml fractions and the pellet were collected and used for HRP activity determination or Western blot analysis.

**Electron Microscopy**—A431 cells were plated 18–24 h before each experiment and were used at 60–80% confluency. CT and CT conjugated to horseradish peroxidase (CT-HRP) were purchased from Sigma. CT adsorbed to 14-nm gold (CT-gold) was prepared exactly as described previously (15). A431 cells were washed in DMEM, 10 mM Hepes containing 0.2% BSA and incubated in this medium for 3 h before each experiment. 2B2318 cells were similarly washed by centrifugation and resuspended in HBSS, and then labeled. Alternatively, unfixed cells were labeled directly in HBSS, and then prefixed with 0.5% glutaraldehyde in 3% paraformaldehyde at room temperature; aldehyde-gold particles were then collected in 50 µs acetylated buffer and processed for Epon sections as described previously (22).

To determine the percentage of plasma membrane occupied by invaginations we used cells that were surface labeled with CT-HRP at 8 °C. The invaginations were arbitrarily classified as follows: <100 nm, i.e. including structures of the size of caveolae; and ≥100 nm, invaginations greater than 100 nm in any direction, i.e. excluding typical caveolea-size structures (see Fig. 3c). The fraction of surface occupied by profiles with these dimensions was determined by intersection counting (23). To determine the percentage of gold particles within the various sized structures, over 400 gold particles were counted for each condition.

In order to detect Thy1, T lymphocytes were incubated in HBSS for 1 h to remove serum, prefixed for 10 min with 0.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, washed for four times each in 1 min in HBSS, and then labeled. Alternatively, unfixed cells were labeled directly after HBSS incubation. Cells were labeled using the rabbit polyclonal anti-Thy1 antibody at 8 °C followed by protein A gold. Both prefixed and unfixed cells were then processed for Epon embedding as described above.

**Capping and Immunofluorescence Microscopy**—For G_{M1}, capping experiments, 1 x 10^6 2B2318 cells were incubated for 30 min on ice with 100 µl of a 10 µg/ml solution of CT in PBS, 0.1% BSA. Cells were washed and incubated on ice for a further 30 min with a 1:50 dilution of rabbit anti-CT antibodies (Sigma). After resuspension in a 1:500 dilution of rhodamine-conjugated AffiniPure donkey anti-rabbit IgG antibodies (Dianova, Hamburg, Germany), capping was induced by incubating cells at 37 °C for 25 min. Cells were then washed with PBS(−), plated on multiwell slides pretreated with 0.05% poly-L-lysine (Sigma), and fixed for 7 min in PBS(−), 3% paraformaldehyde at room temperature; 2-methyl groups were then quenched for 5 min in 50 µs NH_4Cl. After blocking with PBS, 0.1% BSA, Thy1 was detected by treating cells with a 1:500 dilution of rabbit monoclonal anti-Thy1 (Boehringer, Mannheim, Germany) followed by fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (Cappel, Durham, NC) preadsorbed with rabbit IgG coupled to Sepharose. Slides were mounted in Mowiol and analyzed by confocal microscopy.

**RESULTS AND DISCUSSION**

To investigate the relationship between glycolipid-rich complexes and caveolae in lymphocytes, we first examined whether these cells expressed the caveolar marker VIP21-caveolin. Four lymphoid cell lines were chosen for this analysis: the B lymphoma A20, the myeloma Ag8, and the T cells Jurkat and 2B2318. By Western blot, no VIP21-caveolin was detectable in the lymphocyte lysates using the anti-caveolin monoclonal antibody, whereas the characteristic VIP21-caveolin doublet (20–21 kDa) was visible in the control cell lines MDCKII, NIH-3T3, and HeLa (Fig. 1a, left panel). Similarly, no VIP21-caveolin was detected in 2B2318 cells using the affinity-purified rabbit anti-VIP21-N antibody (Fig. 1a, right panel). The absence of caveolin in lymphocytes was confirmed by Northern blot analysis (Fig. 1b). In all lymphoid cell lines tested, the VIP21-caveolin band was not visible even with longer exposures or if lower stringency washes were used.

In the absence of caveolin, we investigated whether glycosphingolipids were detergent-insoluble in 2B2318 cells. In fibroblasts a large proportion of G_{M1} was shown to be detergent-insoluble under conditions where the bulk of membrane proteins and other lipids were solubilized (24). Using a similar procedure, 2B2318 lymphocytes and A431 cells, an epidermoid cell line with numerous caveolae (15), were incubated with CT-HRP or transferrin-HRP and extracted with a buffer containing 1% Triton X-100, and detergent-insoluble complexes were separated from soluble material by centrifugation. With both cell lines approximately 99% of surface-bound transferrin-HRP was released into the supernatant, whereas a large fraction of surface-bound CT-HRP was detergent-insoluble (74% insoluble in 2B2318 cells, 46% in A431 cells).

To further characterize the glycosphingolipid-rich detergent-insoluble complexes separated from high density insoluble material, we prepared them by flotation on a sucrose step gradient as described previously (9, 10). 2B2318 cells were incubated before detergent treatment with CT-HRP to allow detection of G_{M1}; the GPI-anchored protein Thy1 and the transmembrane protein integrin β1 were analyzed by Western blot (Fig. 2). Using this procedure, it was possible to estimate the percentage of each component recovered in the detergent-insoluble complexes under these conditions. As summarized in the graph, a large fraction of Thy1 and of the CT-HRP-labeled G_{M1} was found at the top of the gradient while all integrin β1 remained in the bottom fractions. A similar distribution of G_{M1} and Thy1 was observed
without CT treatment (data not shown). Therefore, G\textsubscript{M1} and Thy1 are present in low density detergent-insoluble complexes in the lymphocytes tested despite the absence of caveolin.

We then investigated whether lymphocytes have surface invaginations with the morphology of caveolae. We used CT, which binds to the ganglioside G\textsubscript{M1}, as a putative marker for caveolae (15). Due to the expression of G\textsubscript{M1}, the T hybridoma 2B2318 was selected for this analysis. Cells were incubated with either CT-HRP or CT-gold. After surface labeling with CT-HRP at 8 °C a uniform labeling of the entire plasma membrane was apparent (Fig. 3a). Although low labeling was observed in some invaginations of the surface, these invaginations were very heterogeneous in size and shape. In contrast to A431 cells, in which invaginations with the characteristic size and shape of caveolae were labeled (inset), very few invaginations with the morphology of caveolae were evident in 2B2318 cells. This was examined in a quantitative fashion using CT-gold. CT-gold was distributed fairly evenly over the lymphocyte surface (results not shown). Approximately 0.6% of the gold was found in invaginations smaller than 100 nm (Table I). These invaginations occupied 0.2% of the plasma membrane, showing that there is not a great enrichment in these structures.

Recent studies showed that antibody labeling of a GPI-anchored protein prior to fixation causes its clustering in caveolae (25). We therefore investigated the distribution of Thy1 with and without a pre-fixation step. After a brief fixation with glutaraldehyde, Thy1 labeling was evenly distributed over the cell surface (Fig. 3b). 0.65% of the gold label was present in invaginations of diameter less than 100 nm. When cells were labeled at 8 °C prior to fixation, gold particles were found clustered within invaginations of various sizes (Fig. 3, c and d). Under these conditions 13% of the gold was present within structures larger than 100 nm, but only 5.8% was found within smaller structures (Table I). This was not the result of trapping molecules during labeling or inadequate washing, as protein A gold in the absence of antibody was not observed in these structures (results not shown). In conclusion, our results show that invaginations with the characteristic morphology of caveolae are extremely rare in 2B2318 lymphocytes. The few small invaginations observed were not highly labeled by caveolar markers.

To further investigate the possible association of G\textsubscript{M1} and Thy1, we performed capping experiments in which immunofluorescence was used to study the distribution of one of the two antigens on the cell surface after capping of the second one. In contrast to their extensive association in detergent-insoluble complexes, no significant redistribution of Thy1 was induced upon G\textsubscript{M1} capping (Fig. 4). Similarly G\textsubscript{M1} appeared evenly distributed after capping of Thy1, consistent with preliminary electron microscopy observations (results not shown).
results suggest that there is little association of G_{M1} and Thy1 in the living cell, despite the bulk of these components associating with detergent-insoluble complexes.

Our results show that VIP21-caveolin is not expressed in lymphocytes and that lymphocytes lack, or have exceedingly low numbers of, caveolae. In this study we defined caveolae by the presence of GPI-anchored proteins and GSLs after surface labeling, the presence of VIP21-caveolin, and by morphology at the ultrastructural level. While detergent insolubility has been used to enrich for caveolar components our results show that detergent insolubility does not correlate with caveolar localization. Consistent with a number of other studies, a large fraction of the GPI-anchored protein Thy1 and of the ganglioside G_{M1} was detergent-insoluble in lymphocytes. In A431 cells caveolae were shown to occupy 7% of the plasma membrane, and approximately 50% of a cholera toxin conjugate was present in caveolae after labeling the cell surface (15). Nevertheless, the fraction of insoluble G_{M1} after cholera toxin surface labeling was similar in lymphocytes and in A431 cells. Similarly, under conditions in which we labeled Thy1 in caveolae of NIH-3T3 cells (results not shown) we found little concentration of Thy1 in caveolae-like invaginations of lymphocytes although patches of labeling were found in heterogeneously sized invaginations. The lack of a correlation between caveolar localization and detergent insolubility is also consistent with our previous studies showing that VIP-36 is not concentrated in caveolae under steady state conditions and yet is largely detergent-insoluble (26). Interestingly, VIP-36 was shown to be concentrated in caveolae after antibody cross-linking at the cell surface. Similarly, recent studies suggested that GPI-anchored proteins are only clustered in caveolae after antibody addition (25), and yet in the absence of antibody-induced clustering they are present in detergent-insoluble complexes in epithelial cells (13). Taken together, these observations show that detergent insolubility cannot correlate with caveolar localization. However, the fact that all these components specifically enter caveolae on antibody-induced cross-linking and under steady state conditions are detergent-insoluble suggests some link between these processes. The fact that both G_{M1} and Thy1 were found in low density fractions after sucrose density centrifugation suggests that both are associated with glycolipid microdomains. However, co-capping experiments suggested that the bulk of G_{M1} and Thy1 were not associated in interconnected structures in living cells. Alternatively, the interaction may have too low stability in the absence of detergents for efficient co-capping. We therefore envisage that detergent treatment might result in the formation of larger insoluble aggregates which, although reflecting the presence of glycolipid-rich domains in vivo, would not correlate exactly with the organization of these domains in the living cell. The lack of association of G_{M1} and Thy1 in lymphocytes might be related to the lack of VIP21-caveolin expression; interestingly, the absence of VIP21-caveolin in the thyroid epithelial cell line FRT has been suggested to be responsible for the impaired interaction of glycosphingolipids and a GPI-anchored protein during their transport to the cell surface (27).

Finally our results suggest that caveolae, as defined by several criteria, are not involved in the well characterized signal transduction pathways in lymphocytes. Several different T and B lymphocyte cell lines, including Jurkat, were shown to be activated by cross-linking of endogenous or transfected Thy1 (28). Activation via anti-Thy1 antibodies induces tyrosine phosphorylation of specific substrates in 2B4 cells, a murine T hybridoma closely related to 2B2318 cells (4, 29). The absence of morphologically defined VIP21-caveolin-positive caveolae in lymphocytes suggests that these structures are not essential for basic cell function and that they are more likely to have a more specialized role. We do not know whether the heterogeneous invaginations that were labeled with antibody-labeled Thy1 could show some functional similarities to caveolae. However, we can conclude that neither VIP21-caveolin nor the characteristic and highly conserved structure of the caveolar domain is required for such a function in lymphocytes.

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