Glycoconjugate Journal 17, 247–252, 2000

Association between GM3 and CD4-Ick complex in human peripheral blood lymphocytes

Maurizio Sorice[1], Tina Garofalo[1], Roberta Misasi[1], Agostina Longo[1], Joanna Mikulak[1], Vincenza Dolo[2], Giuseppe Mario Pontieri[1] and Antonio Pavan[2]*

[1]*Dipartimento di Medicina Sperimentale e Patologia, Universitá di Roma 'La Sapienza', Viale Regina Elena 324, Roma 00161, Italy;* [2]*Dipartimento di Medicina Sperimentale, Università di L'Aquila, Via Vetoio Coppito 2, L'Aquila 67100, Italy*

The aim of this study was to further elucidate our previous observation on molecular interaction of GM3, CD4 and p56^{lck} in microdomains of human peripheral blood lymphocytes (PBL). We analyzed GM3 distribution by immunoelectron microscopy and the association between GM3 and CD4-p56^{lck} complex by scanning confocal microscopy and co-immunoprecipitation experiments. Scanning confocal microscopy analysis showed an uneven signal distribution of GM3 molecules over the surface of human lymphocytes. Nearly complete colocalization areas indicated that CD4 molecules were distributed in GM3-enriched plasma membrane domains. Co-immunoprecipitation experiments revealed that CD4 and p56^{lck} were immunoprecipitated by IgG anti-GM3, demonstrating that GM3 tightly binds to the CD4-p56^{lck} complex in human PBL. In order to verify whether GM3 association with CD4 molecules may depend on the presence of p56^{lck}, we analyzed this association in U937, a CD4 + and p56^{lck} negative cell line. The immunoprecipitation with anti-GM3 revealed the presence of a 58 kDa band immunostained with anti-CD4 Ab, suggesting that the GM3-CD4 interaction does not require its association with p56^{lck}. These findings support the view that GM3 enriched-domains may represent a functional multimolecular complex involved in signal transduction and cell activation.

Keywords: **GM3, gangliosides, microdomains, CD4**

Abbreviations: **GSL, glycosphingolipids; PBL, peripheral blood lymphocytes; GEM, glycosphingolipid-enriched microdomains; LDTI, low density Triton X-100 insoluble fraction; PKC, protein kinase C; TCR, T cell receptor; PBS, phosphate buffered saline; MAb, monoclonal antibody; FITC, fluorescein isothiocyanate; LBPA, lysobisphosphatidic acid; SDS, sodium dodecyl sulfate; TNFR1, tumor necrosis factor receptor 1.**

Introduction

Gangliosides, acidic glycosphingolipids (GSL), are ubiquitous constituents of cell membranes [1], where they show cell type-specific expression patterns. In human mononuclear cells monosialoganglioside GM3 represents the main ganglioside constituent of cell plasma membrane [2]. The distribution of these molecules on lymphocyte plasma membrane has been deeply investigated in the last few years [3,4]. Previous immunofluorescence and immunogold electron microscopical studies revealed a clustered distribution of GM3 molecules on the cell surface of human peripheral blood lymphocytes (PBL), clearly indicating the presence of glycosphingolipid (GM3)-enriched microdomains (GEM) [4,5]. These domains have been identified as low density Triton X-100 insoluble fraction (LDTI) due to their poor solubility in cold nonionic detergents, showing an about 20 fold enrichment of GM3 and cholesterol, as compared to total cell lysates [4].

Gangliosides in these fractions may be involved in modulating signal transduction by GSL-GSL interaction, binding with specific antibody or assembly with signal transducer molecules [5]. The variety of proteins detected in these domains isolated from different cell types is extremely wide. The presence of tyrosine kinase receptors, mono- (Ras, Rap) and heterotrimeric G proteins, Src-like tyrosine kinases (Ick, lyn, fyn), PKC isozymes and GPI anchored proteins [6–8] allows to consider these portions of the plasma membrane as 'glycosignaling domains'. We previously demonstrated that, in lymphocytes, CD4 and p56^{lck}, a member of the Src family of tyrosine kinases, are selectively recovered in GM3-enriched LDTI complexes [4]. The CD4-p56^{lck} complex represents one of the most important receptor systems in the T cell function and CD4 is considered to be the TCR co-receptor in thymic selection, T cell activation and cellular response [9–11]. Most

*To whom correspondence should be addressed: Dr. Antonio Pavan, Dipartimento di Medicina Sperimentale, Università degli Studi di L'Aquila, Via Vetoio, Coppito 2, 67100 L'Aquila, Italy. Tel: 39-862-433683; Fax: 39-862-433523; E-mail: pavan@univaq.it

of the CD4 functions are due to CD4 interaction with $p56^{lck}$ [12]. Interestingly, in human T lymphocytes exogenous GM3 induces CD4 phosphorylation [13], dissociation from $p56^{lck}$ and internalization via endocytic pits and vesicles [14].

In order to better analyze the interaction between GM3 and CD4-$p56^{lck}$ complex, we performed in human peripheral blood lymphocytes and in an histiocytic-monocytic leukemic cell line (U937): a) scanning confocal microscopical analysis of the dual surface distribution of GM3 and CD4 molecules; b) co-immunoprecipitation experiments, using anti-GM3 antibody DH2 [15], in order to verify the association of CD4-$p56^{lck}$ complex with immunoprecipitated GM3 molecules.

Materials and methods

Immunoelectron microscopy

Lymphocytes fixed in formaldehyde (4% in PBS for 2 h at 4°C) were incubated with anti-GM3 mAb GMR6 [16] (kindly provided by Dr Tadashi Tai), for 1 h at 4°C. Cells were fixed with glutaraldehyde (1% in PBS, for 1 h at 4°C) and then incubated with rabbit anti-mouse IgM (Sigma Chem. Co, St Louis, USA) (1:10 in PBS, for 1 h at 4°C); cells were extensively washed and then labeled with colloidal gold (18 nm, prepared by the citrate method) conjugated with protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) for 3 h at 4°C. Control experiments were performed omitting the mAb from the immunolabeling procedure. All samples were post-fixed in osmium tetroxide 1% in Veronal acetate buffer, pH 7.4, for 2 h at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812.

Analysis of GM3-CD4 colocalization on the cell surface of PBL by scanning confocal microscopy

$CD4^+$ lymphocytes (1×10^6 in 1 ml of PBS) were fixed with 4% formaldehyde in PBS for 1 h at 4°C. Cells were then labeled with anti-CD4 mAb (Orthodiagnostic, Rariton, NJ, USA) for 1 hr at 4°C, followed by addition of Texas red-conjugated anti-mouse IgG (Calbiochem, La Jolla, CA). After 3 washes in PBS, cells were incubated with anti-GM3 mAb (GMR6) followed by 3 washes in PBS and addition (30 min at 4°C) of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Sigma Chem Co). Separately, in parallel experiments, cells were directly stained with anti-GM3 mAb before fixing the cells with 4% formaldehyde in PBS. Alternatively, cells were processed for a second formaldehyde fixation immediately after the incubation with anti-GM3 mAb and before the secondary antibody. Both fixation procedures did not affect the ganglioside distribution on the cell surface, as already reported [4]. Cells were finally washed three times in PBS and then mounted upside down onto a glass slide in 5 ml of glycerol/Tris-HCl pH 9.2. The coverslips were sealed with nail varnish to prevent evaporation and stored at 4°C before imaging. The images were acquired through a confocal laser scanning microscope (Sarastro 2000, Molecular Dynamics) equipped with a Nikon Optiphot microscope (objective 60/1.4 oil) and an Argon Ion Laser (25 mW output). Simultaneously, the green (FITC) and the red (Texas Red, which reduces greatly overlapping) fluorophores were excited at 488 nm and 518 nm. Acquisition of single FITC stained samples in dual fluorescence scanning configuration did not show contribution of green signal in red. Images were collected at 512×512 pixels (0.08 μm/pixel lateral dimension, 0.48 μm/pixel axial dimension). Serial optical sections were assembled in Depth-Coding mode. Acquisition and processing were carried out using Image Space software (Molecular Dynamics).

Isolation of low-density Triton-insoluble complexes (LDTI)

LDTI complexes were isolated as previously described [17]. Briefly, 5×10^8 U937 cells were washed and resuspended in 2 ml MES-buffered saline (MBS: 25 mM MES, pH 6.5, 0.15 M NaCl) containing 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride and homogenized with 10 strokes of a Dounce homogenizer, adjusted to 40% sucrose and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was then placed above the lysate, and centrifuged at 39,000 rpm for 16 h at 4°C in a SW41 rotor (Beckman Inst., Palo Alto, CA). The visible band migrating at 20% sucrose was harvested and washed twice with MBS at 14,000 rpm for 30 min at 4°C.

Co-immunoprecipitation of GM3 and transducer molecules in PBL and U937 cells

Co-immunoprecipitation of GM3 and proteins was performed according to Iwabuchi et al. [18]. Briefly, human PBL or U937 cell were lysed in lysis buffer (20 mM HEPES, pH 7.2/1% Nonidet P-40/10% glycerol/50 mM NaF/1 mM phenylmethylsulphonyl fluoride/1 mM Na_3VO_4/10 μg of leupeptin per ml). Cell free lysates (containing 20–25 μg of protein) or Triton-insoluble fraction (containing 20–25 μg of protein) were mixed with protein A-Sepharose beads and stirred by a rotary shaker for 2 h at 4°C to preclear nonspecific binding. After centrifugation ($500 \times g$ for 1 min), the supernatant was added with 20 μl of anti-GM3 mAb DH2 (IgG3) ascites [15] (kindly provided by Dr Alessandro Prinetti) or with 20 μl of mouse anti-lysobisphosphatidic acid (LBPA) mAb (IgG) (kindly provided by Dr Jean Gruenberg) (19), 200 μg/ml, as a negative control. The mixtures were placed overnight in a rotary mixer at 4°C, added with protein A-sepharose beads and placed again in a rotary mixer for 2 h. Beads were washed three times with PBS containing 0.01% Tween 20, by brief weak centrifugation ($500 \times g$ for 1 min) and then suspended with 100 μl of sample buffer with mercaptoethanol, heated to 95°C for 3 min, and centrifuged ($1000 \times g$ for 2 min). The supernatants were electrophoretically transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) after SDS-PAGE with 10% polyacrilamide gels and then probed with polyclonal

anti-CD4 (Santa Cruz Biotechnologies, Santa Cruz, CA) and, in PBL, with anti-p56^{lck} Ab (Santa Cruz). Bound antibodies were then visualized with peroxidase-conjugated anti-rabbit IgG (Sigma Chem Co), followed by sodium nitroprusside staining.

Results

Immunoelectron microscopic analysis

To analyze the distribution of GM3, formaldehyde fixed PBL were immunolabeled with anti-GM3 mAb (GMR6) followed by anti-IgM antibodies and by protein A-colloidal gold. Immunoelectron microscopic observations showed an uneven distribution of GM3 on the lymphocyte plasma membrane (Figure 1); the gold immunolabeling appeared in small clusters and localized either over the microvilli or over the non villous portion of the membrane. Gold clusters were also observed inside both uncoated and coated pits (not shown). Our observations confirmed that the immunogold clustering represents the native distribution of GM3 molecules over lymphocyte plasma membrane. Immunogold labeling quantitation, determined as gold clusters/μm length of plasma membrane was performed and reported in Table 1.

Figure 1. Immunolabeling of GM3 molecules on PBL plasma membrane. The surface distribution of the immunogold particles is not uniform. Clusters of variable size are equally localized over the microvilli and over the nonvillous portion of the plasma membrane. Magnification: × 25.500; bar: 0.5 μm.

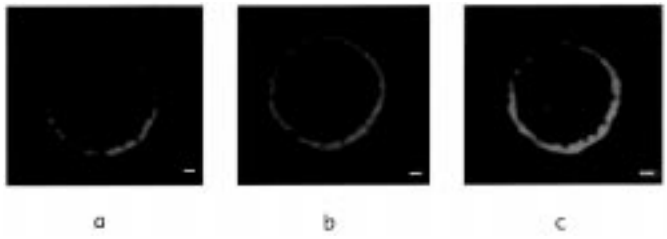

Figure 2. Scanning confocal microscopic analysis of GM3-CD4 association on PBL plasma membrane. Cells were labeled with anti-CD4 mAb, followed by the addition of Texas red-conjugate anti-mouse IgG. After washing with PBS cells were incubated with anti-GM3 (GMR6), followed by the addition of goat anti-mouse IgM conjugated with fluorescein (FITC). Magnification: × 1000; bar 1 μm. Lane a: cells stained with anti-GM3, followed by the addition of goat anti-mouse IgM conjugated with fluorescein; Lane b: cells stained with anti-CD4, followed by the addition of goat anti-mouse IgG conjugated with Texas red; Lane c: dual immunolabeling of anti-GM3 (green) and anti-CD4 (red). Colocalization areas are stained in yellow.

Analysis of the association between GM3 and CD4 in human PBL

In order to study the possible GM3-CD4 interaction in human PBL, we analyzed their distribution on the plasma membrane (Figure 2a, b). The results by scanning confocal microscopy revealed that most of the cells showed an uneven signal distribution of ganglioside molecules over the cell surface (Figure 2b). In order to determine the possible association between CD4 and GM3, we superimposed the double immunostaining of anti-CD4 and anti-GM3. The merged image of anti-CD4 and anti-GM3 staining revealed yellow areas, resulting from overlap of green and red fluorescence, as reported [20, 21]. They correspond to nearly complete colocalization areas, indicating that CD4 molecules were mainly, but not exclusively, localized in membrane domains enriched with GM3 molecules (Figure 2c).

Co-immunoprecipitation of GM3, CD4 and p56^{lck} in human PBL

Lysates from human PBL were immunoprecipitated with anti-GM3 mAb DH2, followed by protein A-Sepharose beads. GM3 and its possible complex with associated proteins were eluted from the beads and subjected to Western blotting. On the basis of our previous demonstration that GM3 is highly enriched in LDTI complexes and CD4 and p56^{lck} are selectively recovered in the same fractions [4], we analyzed the association between GM3 and CD4-p56^{lck} complex, subjecting the blot membrane to a rabbit polyclonal anti-CD4 or anti-p56^{lck} Ab. With this approach, CD4 (Figure 3a)

Table 1. Quantitative analysis of GM3 clusters on lymphocyte plasma membrane

	Total length (μm)	*Total occupied by clusters (μm)*	*Average length of cluster size (μm)*	*Range of length of cluster size (μm)*
Length (μm); (10*)	310.8	85.4	0.16 ± 0.03	0.05–0.32

n. clusters/cell (10*): 52 ± 5
*: Number of lymphocyte plasma membranes analyzed in conventional thin sections.

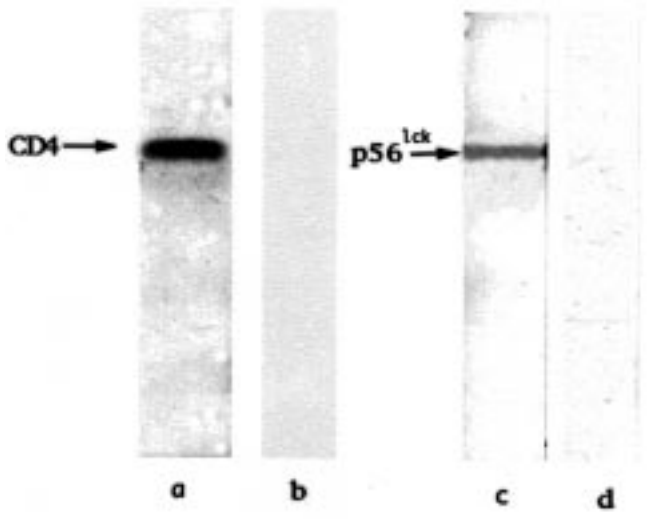

Figure 3. Co-immunoprecipitation of GM3, CD4 and $p56^{lck}$ in human PBL: Western blot analysis of GM3 immunoprecipitate. Human PBL were lysed in lysis buffer. Cell-free lysates were normalized for proteins and immunoprecipitated with IgG anti-GM3 mAb (DH2). Proteins from the immunoprecipitate were separated on 10% SDS-PAGE and probed with the anti-CD4 or anti-$p56^{lck}$ polyclonal Ab. Lane a: GM3 immunoprecipitate, reactivity with anti-CD4 Ab; Lane b: LBPA immunoprecipitate, reactivity with anti-CD4 Ab; Lane c: GM3 immunoprecipitate, reactivity with anti-$p56^{lck}$ Ab; Lane d: LBPA immunoprecipitate, reactivity with anti-$p56^{lck}$ Ab.

and $p56^{lck}$ (Figure 3c) were detected on Western blotting. In control samples the immunoprecipitation with an irrelevant Ab, mouse anti-LBPA mAb, under the same condition, did not result in detectable levels of CD4 or $p56^{lck}$ (Figure 3b and d, respectively).

Co-immunoprecipitation of GM3 and CD4 in U937 cells

In order to verify whether CD4 association with GM3 molecules may depend on the presence of $p56^{lck}$, we analyzed this complex in U937, a CD4+ and $p56^{lck}$ negative cell line.

Lysates and the LDTI fraction from U937 were immunoprecipitated with IgG anti-GM3 mAb (DH2) and protein A-Sepharose beads, as above. GM3 and its possible complex with associated transducer proteins were eluted from the beads and subjected to Western blotting with rabbit polyclonal anti-CD4. It revealed a band corresponding to 58 kDa (Figure 4a). The same band was detected in the LDTI fraction (fraction 5) (Figure 4c). In control samples the immunoprecipitation with an irrevelant Ab, mouse anti-LBPA mAb, under the same condition, did not result in detectable levels of any protein (Figure 4b). These findings demonstrate that, at least in an histiocytic monocytic cell line, the GM3-CD4 interaction is independent of CD4-$p56^{lck}$ association, suggesting the $p56^{lck}$ needless role in clustering of GM3-CD4 molecules.

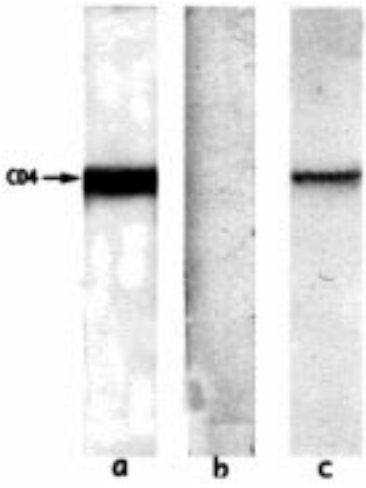

Figure 4. Co-immunoprecipitation of GM3 and CD4 in U937 cells: Western blot analysis of GM3 immunoprecipitate. Cells were lysed in lysis buffer. Cell-free lysates and LDTI fraction were normalized for proteins and immunoprecipitated with IgG anti-GM3 mAb (DH2). Proteins from the immunoprecipitate were separated on 10% SDS-PAGE and probed with the anti-CD4 Ab. Lane a: GM3 immunoprecipitate, reactivity with anti-CD4 Ab; Lane b: LBPA immunoprecipitate, reactivity with anti-CD4 Ab; Lane c: GM3 immunoprecipitate in the LDTI fraction (fraction 5), reactivity with anti-CD4 Ab.

Discussion

In this report we analyzed the clustered distribution of GM3 on lymphocyte plasma membrane and its association with CD4-$p56^{lck}$ complex. Our immunogold observations confirmed the existence of GM3-enriched plasma membrane microdomains characterized by different length of occupied membrane (range 0.05–0.32 μm). The average cluster length appeared to be around 0.2 μm which seems to correspond to rafts of intermediate size [22], previously observed in NIH3T3 [23], and identified as microdomains enriched in liquid-ordered phase lipids and cholesterol and containing up to about 600 proteins. It is well known that rafts may function to concentrate or segregate different proteins in lateral domains of the membrane to form the 'glycosignaling domain', functionally involved in modulation of tyrosine kinase activities, cell adhesion and cell-cell interation [24]. Following our previous observation that in human lymphocytes GM3, CD4, and $p56^{lck}$ are selectively recovered in the same microdomains of the plasma membrane [4], we now provide evidence that GM3 tightly binds to the CD4 antigen in U937 cells and to CD4-$p56^{lck}$ complex in human PBL. In different cell systems a close association of specific gangliosides with specific transducer proteins has been described [5], although the precise mechanism involved remains to be elucidated. In this concern, it has been hypothesized that interaction of ganglioside with some transducers (e.g., c-Srk, Rho) may be due to the presence of aliphatic chain (fatty acyl or farnesyl group) linked to the transducer, although other transducers

present in glycosignaling domains (e.g., FAK, Ras) may require different mechanisms [5]. High affinity SDS-resistant ganglioside-protein-interactions have been reported in different cell types and exert relevant functional effects. GM1 and Trk A kinase in PC12 cells strongly enhance neurite outgrowth and neurofilament expression [25], nonreceptor Src family tyrosine kinase Lyn and α-Gal-GD1b in rat basophilic leukemia cells play an important role in receptor-mediated signal transduction [26] and Lyn and GD3 in rat brain are involved in neuritogenesis and synaptic formation [27]. In addition, SDS-resistant complexes between GM3 and prosaposin on NS20Y neuroblastoma cell plasma membrane are structural components of the prosaposin receptor, involved in extracellular signal-regulated protein kinase (ERK) phosphorylation and cell differentiation [21].

In the present study the GM3-CD4 association was demonstrated by a novel approach, showing that CD4 was co-immunoprecipitated by an IgG anti-GM3 (DH2) [15]. This procedure represents, in our hand, a valuable tool that led us to clarify the role of GM3 as a transducer molecule, with the cooperation of specific proteins. This approach was validated by the studies of Yamamura [28] and Iwabuchi [18], who revealed that in B16 melanoma cells the DH2 antibody immunoprecipitated multiple signal transducer molecules, such as c-Src, Rho and FAK. Furthermore, scanning confocal microscopical images supported the existence of GM3 enriched microdomains on the surface of intact PBL, where CD4 molecules were mainly, but not exclusively, colocalized with GM3. A similar finding was observed in NS20Y neuroblastoma cells showing a selective colocalization of prosaposin with GM3 molecules [21].

However, all these findings do not prompt to conclude whether GM3 binds CD4 directly or through interaction with other unidentified protein(s). In this study we demonstrated that GM3-CD4 interaction can occur in the absence of $p56^{lck}$, as observed in U937 $p56^{lck}$ negative cells. In this cell line the possibility of GM3 interaction with other GEM associated proteins, such as CD14 [8], fyn [29], TNFR1 [29] and CD48 [30] requires further investigation. In this concern, we hypothesize that immunoprecipitation by anti-GM3 Ab is like to pull out a 'clod' of plasma membrane, due to tight and weak binding associations among GM3 molecules and different proteins, which may represent the multimolecular signaling complex.

In conclusion, both morphological and biochemical analyses demonstrate the existence of GM3 microdomains on lymphocyte plasma membrane, where the presence of ganglioside-protein(s) interactions strongly supports the functional role of these rafts as structural components of membrane multimolecular signaling complexes.

Acknowledgements

We thank professor Tadashi Tai (Tokyo Metropolitan Institute of Medical Science) for providing us with the IgM anti-GM3 GMR6, and Dr Alessandro Prinetti, Department of Medical Chemistry and Biochemistry, University of Milan, for providing us the IgG anti-GM3 DH2. This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), Consiglio Nazionale delle Ricerche (CNR) (n. 98.00515.CT04) and Ministero del lavoro e della Previdenza n. 792.

References

1 Hannun YA, Bell RM, Functions of sphinolipids and siphingolipid breakdown products in cellular regulation. *Science* **243**, 500–7 (1989).
2 Kiguchi K, Henning-Chubb BC, Huberman E, Glycosphingolipid patterns of peripheral blood lymphocytes, monocytes and granulocytes are cell specific. *J Biochem* **107**, 8–14 (1990).
3 Fra AM, Williamson E, Simons K, Parton RG, Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J Biol Chem* **269**, 30745–8 (1994).
4 Sorice M, Parolini I, Sansolini T, Garofalo T, Dolo V, Sargiacomo M, Tai T, Peschle C, Torrisi MR, Pavan A, Evidence for the existence of ganglioside-enriched plasma membrane domains in human peripheral lymphocytes. *J Lipid Res* **38**, 969–80 (1997).
5 Hakomori S, Handa K, Iwabuchi K, Yamamura S, Prinetti A, New insights in glycosphingolipid function: "glycosignalig domain", a cell surface assembly of glycoshpingolipids with signal transducer molecules, involved in cell adhesion coupled with signaling. *Glycobiology* **8**, xi–xix (1998).
6 Parolini I, Sargiacomo M, Lisanti MP, Peschle C, Signal transduction and glycophosphatidylinositol-linked proteins (LYN, LCK, CD4, CD45, G proteins, and CD55) selectively localize in Triton-insoluble plasma membrane domains of hman leukemic cell lines and normal granulocytes. *Blood* **87**, 3783–94 (1996).
7 Stefanova I, Horejsi V, Associationof the CD59 and CD55 cell surface glycoproteins with other membrane molecules. *J Immunol* **147**, 1587–92 (1991).
8 Horejsi V, Drbal K, Cebecauer M, Cerny J, Brdicka T, Angelisova P, Stockinger H, GPI-microdomains: a role in signalling via immunoreceptors. *Immunology Today* **20**, 356–61 (1999).
9 Blue ML, Hafter DA, Craig KA, Levine H, Schlossman SF, Phosphorylation of CD4 and CD8 molecules following T cell triggering. *J Immunol* **139**, 3949–54 (1987).
10 Gay D, Buus S, Pasternak J, Kappler J, Marrak P, The T-cell accessory molecule CD4 recognizes a monomorphic determinant on isolated la. *Proc Natl Acad Sci USA* **85**, 5629–34 (1988).
11 Veillette A, Bookman NA, Horak EN, Samelson LE, Bolen JB, Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature* **338**, 257–9 (1989).
12 Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, Schlossman SF, The CD4 receptor is complexed in detergent lystaes to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc Natl Acad Sci USA* **85**, 5190–5 (1988).
13 Garofalo T, Sorice M, Misasi R, Cinque B, Giammatteo M, Pontieri GM, Cifone MG, Pavan A, A novel mechanism of CD4

down-modulation induced by monosialoganglioside GM3. *J Biol Chem* **273**, 35153–60 (1998).
14 Sorice M, Pavan A, Misasi R, Sansolini T, Garofalo T, Lenti L, Pontieri GM, Frati L, Torrisi MR, Monosiloganglioside GM3 induces CD4 internalization in humanperpheral blood T lymphocytes. *Scand J Immunol* **41**, 148–56 (1995).
15 Dohi T, Nores G, Hakomori S, Cancer Res **48**, 5680–5 (1988).
16 Kotani M, Ozawa H, Kawashima I, Ando S, Tai T, Generation of one set of monlonal antbodies specific for a-pathway ganglioseries gangliosides. *Biochim Biophys Acta* **1117**, 97–103 (1992).
17 Sargiacomo M, Sudol M, Tang ZL, Lisanti MP, *J Cell Biol* **122**, 789–807 (1993).
18 Iwabuchi K, Yamamura S, Prinetti A, Handa K, Hakomori S, GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate-carbhydrate interaction in mouse melanoma. *J Biol Chem* **273**, 9130–8 (1998).
19 Kobayashi T, Stang E, Fang KS, de Moerloose P, Parton RG, Gruenberg J, A lipid association with the antiphospholipid syndrome regulates endosome structure and function. *Nature* **392**, 193–7 (1998).
20 Okamoto Y, Ninomiya H, Miwa S, Masaki T, Cholesterol oxidation switches the internalization pathway of endothelin receptor A from caveolae to clathrin-coated pits in chinese hamster ovary cells. *J Biol Chem* **275**, 6439–46 (2000).
21 Misasi R, Sorice M, Garofalo T, Griggi T, Campana WM, Giammatteo M, Pavan A, Hiraiwa M, Pontieri GM, O'Brien JS, *J Neurochem* **71**, 2313–21 (1998).
22 Jacobson K, Dietrich C, Looking at lipid rafts? Trends *Cell Biol* **9**, 87–91 (1999).
23 Visco V, Lucania G, Sansolini T, Dolo V, Garofalo T, Sorice M, Frati L, Torrisi MR, Pavan A, Expression of GM3 microdomains on the surfaces of murine fibroblasts correlates with inhibition of cell proliferation. *Histochem Cell Biol* **113**, 43–50 (2000).
24 Simons K, Ikonen E, Functional rafts in cell membranes. *Nature* **387**, 569–72 (1997).
25 Mutoh T, Tokuda A, Miyadai T, Hamaguchi M, Fujiki N, Ganglioside GM1 binds to the Trk protein and regulates receptor functions. *Proc Natl Acad Sci USA* **92**, 5087–91 (1995).
26 Minoguchi K, Swaim WD, Berenstein EH, Siraganian RP, Src family tyrosine kinase p53/p56^{lyn}, a serine kinase and FcεRI associate with α-galactosyl derivates of ganglioside GD1b in rat basophilic leukemia RBL-2H3 cells. *J Biol Chem* **269**, 5249–54 (1994).
27 Kasahara K, Watanabe Y, Yamamoto T, Sanai Y, Association of Src family tyrosine klinaser lyn with hahlioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolypid in caveolae-like domains. *J Biol Chem* **272**, 29947–53 (1997).
28 Yamamura S, Handa K, Hakomori S, A close association of GM3 with c-Src and Rho in GM3-enriched microdomains at the b16 melanoma cell surface membrane: a preliminary note. *Biochem Biophys Res Commun* **236**, 218–22 (1997).
29 Ko YG, Lee JS, Ahn JH, Seo JS, TNF-α-mediated apoptosis is intiated in caveolae-like domains. *J Immunol* **162**, 7217–23 (1999).
30 Moran M, Miceli MC, Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. *Immunity* **9**, 787–96 (1998).