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ASSOCIATION OF THE GPI-ANCHORED LEUCOCYTE SURFACE GLYCOPROTEINS WITH GANGLIOSIDE GM3

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Summary. The major glycolipid co-immunopurifying with the glycosylphosphatidylinositol-anchored leucocyte surface glycoprotein CD59 from detergent lysates of human T cell lines HPB ALL, Jurkat and myeloid line HL-60 was identified as the glycosphingolipid GM3. Monoclonal antibodies to GM3 immunoprecipitated the same large detergent-resistant, protein-tyrosine kinase containing "GPI-complexes" as antibodies to several GPI-anchored proteins. Therefore GM3 is another component of these large membrane complexes potentially involved in signalling through GPI-anchored receptors or through some glycolipids.

Recently we have described the existence of large, detergent-resistant complexes containing several GPI-anchored leucocyte surface glycoproteins, protein-tyrosine kinases and (glyco)lipids (1-3). We hypothesized that these complexes detectable after cell solubilization by NP-40 or similar detergents reflect the existence of specific membrane microdomains in the plasma membrane of leucocytes involved in the otherwise somewhat puzzling ability of the GPI-anchored receptors and glycolipids to transduce signals through the membrane (4, 5). Similar detergent-resistant membrane structures of specific (glyco)lipid and protein composition were described in epithelial cells (6, 7) and implicated in phenomena such as a kind of endocytosis (8) and membrane vesiculation (9). In the present communication we report that the ganglioside GM3 is the major glycolipid associated with these "GPI-complexes" in at least two human T-cell lines and one myeloid cell line and that mAbs to GM3 effectively precipitate these PTK-containing complexes.

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Abbreviations: GPI-glycosylphosphatidylinositol; mAb - monoclonal antibody; GSL - glycosphingolipid; PTK - protein-tyrosine kinase.

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MATERIALS AND METHODS

Antibodies. MAbs MEM-43 (recognizing the human GPI-anchored glycoprotein CD59) and MEM-57 (against human CD3) were described earlier (10, 11). MEM-97 (CD20) was prepared in our laboratory. MAb M2590 (12, 13) recognizing the GM3 ganglioside (II^{3}NeuAc-lactosyl ceramide) and reactive also with other α 2-3 terminally sialylated gangliosides of the neolacto series, was obtained directly from Dr. S. Taniguchi (Chiba University, Chiba, Japan) and in part from the CDw65 panel of the 5th International Workshop on Human Leukocyte Differentiation Antigens. The other anti-GM3 mAb DH2 used for immunoprecipitation was kindly provided by Dr. S. Hakomori (The Biomembrane Institute, Seattle, WA).

Cell lines. The HPB-ALL thymoma cell line was originally obtained from the laboratory of Professor J.L.Strominger (Harvard University, Cambridge, MA), T cell line Jurkat and myeloid cell line HL-60 were from the collection of Dr. J. Stöckbauer (Institute of Haematology and Blood Transfusion, Praha). The cells were grown in RPMI medium supplemented with 10% calf serum.

Glycolipids. A monosialoganglioside fraction was obtained from a bulk of unseparated human leukocytes essentially as described (14). For purification of GM3, the monosialogangliosides were separated by HPLC similarly as described (14) using a column (16 x 500 mm, Knauer, Berlin, FRG) filled with Lichrosorb Si 60 5 μm silica (Merck, Darmstadt, FRG). A linear gradient was chosen ranging from 2-propanol-hexane-water 55/42/3 (V/V) to 70/10/20 (V/V) in 600 minutes at a flow rate of 1.5 ml/min. 200 fractions were collected. The GM3 containing fractions (104-114), which still showed an unidentified contaminating substance, were further purified using the same column and a linear gradient ranging from chloroform-methanol-water 82.6/16.4/1 to 40/50/10 (V/V) in 400 min at a flow rate of 2 ml/min. Pure GM3 was found in the fractions 112-131. Identity and purity of this ganglioside was confirmed by fast atom bombardment mass spectrometry, degradation with sialidase to lactosylceramide and finally by immunostaining with mAb M2590.

Isolation of the glycolipids associated with the "GPI-complexes". The materials used subsequently for glycolipid analysis were obtained by the solid phase immunoisolation technique (15) modified as described earlier (2). Briefly, the wells of polyvinylchloride microculture plates (Flow ICN, Meckenheim, FRG) were coated with rabbit anti-mouse Ig followed by 50x diluted ascitic fluids containing the anti-CD59 or anti-CD3 (negative control) mAbs and remaining adsorption-active sites were blocked by gelatine. Cells (50 x 10^6/ml) were solubilized for 30 min. on ice in a lysis solution containing 150 mM NaCl, 20 mM Tris-HCl pH 8.2, 1 mM phenylmethylsulfonylfluoride, 5 mM iodoacetamide and 1% detergent NP-40 (Fluka, Buchs, Switzerland), nuclei and other insoluble materials removed by low speed centrifugation (3000 xg, 10 min) and the supernatant was pipetted into the mAb-coated wells (50 μl/well). The plates were incubated overnight on ice, washed 3-times by phosphate-buffered saline and once by water, briefly dried, the lipidic materials eluted with chloroform/methanol (2:1) and solvents removed by evaporation under the stream of nitrogen. In total, the lysate of 2.5 x 10^8 cells was used in each case. However, the binding capacity of the mAb-coated wells is known to be low and therefore the yield of the isolated antigens (and associated substances) is in our experience probably less than 20%.

Analysis of the lipid constituents. The materials obtained as described in the previous paragraph were taken up in 5 ml 0.1 M aqueous KCl under sonication and loaded immediately onto a 1 x 10 cm glass column filled with 360 mg Sep-Pak C-18 reversed phase material (Millipore, Eschborn, FRG) (16) pretreated by washing successively with chloroform, methanol, water, and equilibrated with 0.1 M aqueous KCl. The first KCl eluate was applied

again in order to ensure a complete adsorption (16). Salts were removed by rinsing the column with 20 ml water, the lipid fraction then eluted with 20 ml methanol. The methanol eluate was evaporated using a gentle stream of nitrogen, dissolved under sonication in 30 µl of chloroform-methanol 2/1(V/V) and centrifuged (10 min. at 4000xg) to remove chloroform-methanol insoluble material. Aliquots of the clear supernatants were applied as 5 mm bands on silica nano TLC plates (Merck) and developed in chloroform-methanol-water 50/40/10 (V/V) containing 0.05% (W/V) $CaCl_2$ for 40 min at ambient temperature. After drying, lipids were monitored consecutively on the same plate using first UV detection at 200 nm (reflection mode) with a CS-9001PC Scanner (Shimadzu, Düsseldorf, FRG) then fluorescence detection after spraying the plate with 0.01% (W/V) Primulin (Direct Yellow 59, Sigma, München, FRG) in acetone-water 8/2 (V/V) with subsequent scanning using the same scanner with an additional fluorescence equipment (xenon lamp, excitation wavelength 369 nm, fluorescence filter No. 3) and finally carbohydrate staining using the DIG staining method (17). To increase sensitivity, a 1:200 dilution of the DIG hydrazide and a 1:100 dilution of the phosphatase-labeled anti-DIG antibody were used, respectively (18). Chemicals used for this method were purchased from Boehringer, Mannheim, FRG, with the exception of 5-chloro-4 bromo-indolyl-3phosphate p-toluidine salt (BCIP) which was obtained from Biomol, Hamburg, FRG. Immunostaining was performed as described earlier (19) with modifications (20).

Immunoprecipitation and in vitro kinase assays. Cell surface radioiodination, immunoprecipitation of the radiolabeled antigens, gel chromatography of NP-40 lysates on Sepharose 4B and in vitro kinase assays on the immunoprecipitates were all performed as described in detail in our previous paper (2).

RESULTS

Identification of the CD59-associated glycolipids. The lipids extracted from the materials immunoisolated on immobilized CD59 mAb (or CD3 controls) from NP-40 lysates of human HPB-ALL, Jurkat and HL-60 cell lines were analyzed as described in Materials and Methods by TLC. The fluorescence patterns after Primulin staining are shown in Fig. 1. Similar profile was obtained also after DIG staining, except that the Primulin staining was more sensitive in the detection of nonpolar glycolipids. The broad group of peaks observed both in the negative controls (CD3) and CD59 associated materials (denoted as I in Fig. 1) is frequently observed in crude lipid extracts and is known to consist of free ceramides, cholesterol, triglycerides, nonpolar phospholipids and sulfatides. This material observed also in the negative controls was obviously nonspecifically adsorbed to the immunosorbent (mAb-coated plastic wells) and therefore it was not analyzed further. However, it should be noted that the CD59 associated material contained an additional sharp peak at the lower end of "I" (Fig. 1). Up to now, this peak could not be separated from the nonspecifically adsorbed material and therefore has not been analyzed. In contrast to the CD3 controls, the materials eluted from the CD59 immunosorbent wells contained a doublet of zones (designated II in Fig. 1A) which was stained with DIG in a manner typical for glycolipids (not shown) and

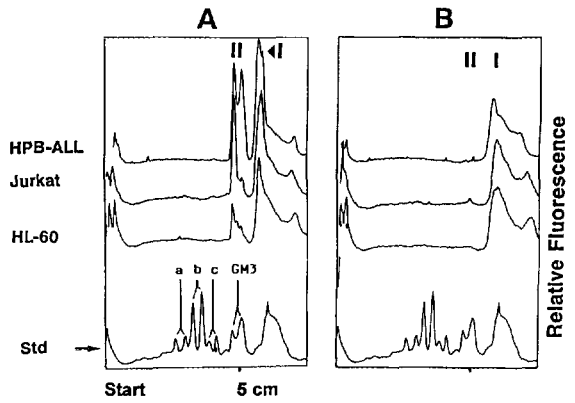

Figure 1. Fluorescence pattern of lipid constituents detected in immunoprecipitates from human cell lines HPB-ALL, Jurkat and HL-60. A. TLC separated lipids from the CD59 precipitates of the 3 human lines. B. TLC separated lipids from the CD3 control precipitates. The standard glycolipid mixture at the bottom was the monosialoganglioside fraction of unseparated human leukocytes and contained at the indicated positions double bands of: a. 2-3 sialosyl-lacto-N-norhexaosylceramide; b. 2-6 sialosyl-neolactotetraosylceramide; c. 2-3 sialosyl-neolactotetraosylceramide and GM3 (2-3 sialosyl-lactosylceramide). Separation and detection as described in Materials and Methods. The nature of peaks I and II is discussed in the text.

co-migrated with the GM3 standard. The identity of this standard as GM3 was confirmed as described in Materials and Methods, including immunostaining with the GM3 specific mAb (Fig. 2). A small amount of the doublet II comigrating with GM3 was also found in the material immunoisolated from the HL-60 cells by means of mAb to CD59 (Fig. 1A). In this case GM3 could not be detected by the GM3 specific antibody (not shown), presumably because of the lower amount of this antigen as compared to the materials obtained from Jurkat and HPB-ALL. Traces of other glycolipid antigens (CDw65, CD15) were also detected by immunostaining in the CD59-associated material obtained from HL-60 cells (not shown). To exclude the possibility that GM3 was co-isolated with CD59 due to the cross-reactivity of the anti-CD59 mAb MEM-43 with GM3, the mixture of glycolipid standards was immunostained with MEM-43; the result was negative (not shown). Taken together, GM3 was clearly detected as a specific component co-purifying with CD59 at least in the HPB-ALL and Jurkat cells.

MAbs to GM3 immunoprecipitate the "GPI-complexes" containing PTK's. After we identified GM3 as the major glycolipid associated with the CD59, we tested whether the anti-GM3 mAb immunoprecipitates the "GPI-complexes" similarly to mAbs to CD59 or other GPI-anchored antigens. Indeed, the results of the in vitro kinase assays on GM3 and CD59

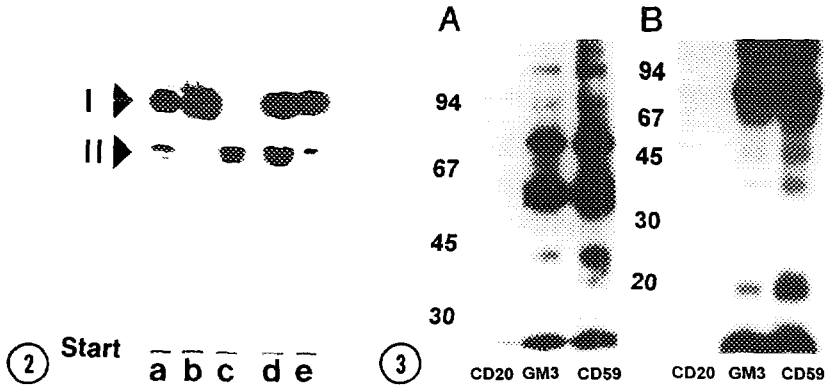

Figure 2. Immunostaining pattern of the lipid constituents in Jurkat and HPB-ALL immunoprecipitates using the GM3 specific antibody M2590. Lanes a and b, lipid constituents of the CD59 and CD3 precipitates from Jurkat, respectively; lane c, 1 μg of GM3 standard; lanes d and e, lipid constituents of the CD59 and CD3 precipitates from HPB-ALL, respectively. Solvent and separation conditions as in Fig. 1. The staining of zone I was nonspecific (i.e., it was observed also when irrelevant mAbs were used for staining). Zone II was stained specifically by M2590 in a concentration dependent manner, comigrated with GM3 standard, and was also stained by DIG being specific for carbohydrate-containing molecules.

Figure 3. Immunoprecipitation of the "GPI-complexes". A. In vitro phosphorylation of the materials immunoprecipitated on MEM-43 (CD59), MEM-97 (CD20, negative control), DH2 *(GM3) and MEM-43 (CD59) mAbs from the NP-40 lysate of HPB ALL cells. The proteins* labeled in the immunoprecipitate by the in vitro kinase reaction with ^{32}P were analyzed by SDS PAGE and autoradiography (positions of mol. wt. standards in kDa are indicated); similar result as with DH2 was obtained also with the other GM3 mAb M2590 (not shown). B. Immunoprecipitation of the "GPI-complexes" from the NP-40 lysate of ^{125}I-labeled HPB-ALL cells using the same mAbs as in A. The immunoprecipitates were analyzed by SDS PAGE and autoradiography. Similarly negative as CD20 were also several other negative controls (e.g., CD3, CD43, CD45).

immunoprecipitates were essentially identical (Fig. 3A). Also, the profiles of the protein zones coprecipitated from the lysate of ^{125}I-labeled HPB-ALL cells by means of mAbs to GM3 and CD59 were similar (Fig. 3B). The complexes shown in Fig. 3 were immunoprecipitated by the CD59 and GM3 mAbs only from the void volume fractions of Sepharose 4B column indicating very large size of these complexes, as described for the GPI-anchored glycoproteins earlier (2) (not shown). These results are fully in agreement with the idea that CD59 and GM3 are jointly components of the large "GPI-complexes" containing also PTK's of the src family (2, 3).

DISCUSSION

Association of glycolipids with GPI-anchored proteins and PTK's in the membranes of leucocytes was already indicated by the results of our previous studies (1-3). In this communication we present direct evidence that the major glycolipid present in these large detergent-resistant complexes in the two T-cell lines examined is the glycosphingolipid GM3. This may not be surprising as GM3 is the major GSL of activated T-cells and T cell lines. Interestingly, small amounts of GM3 were found also in the GPI-complexes isolated from the myeloid cell line HL-60. Our results lend further support to the idea that at least a fraction of the leucocyte surface GPI-anchored (glyco)proteins are present in relatively detergent-resistant areas of the membrane (membrane microdomains) enriched in specific (glyco)lipids and associated with some intracellular proteins including PTK's. It is conceivable that the GPI-anchored proteins may have a tendency to associate with such microdomains due to their structural similarity to glycolipids. There are indications that interactions between the glycolipids and cholesterol are critical for maintaining integrity of such membrane domains in other cell types (21, 22).The central question that remains to be solved is what is the "native" structure of these "GSL-GPI-PTK-domains" and to what extent is it affected by the solubilization procedure, especially by the nature of the detergent used. There are clear indications that these membrane microdomains do exist in the membranes, based on microscopic observations of clustered distribution of GPI-anchored proteins (7, 8, 21), but it is not clear whether some of their components are not lost during detergent extraction. In any case, the existence of these PTK-associated microdomains may explain at least to some extent the signalling capacity of GPI-anchored membrane proteins and of some glycolipids.

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