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THE NATURE OF LARGE NONCOVALENT COMPLEXES CONTAINING GLYCOSYL-PHOSPHATIDYLINOSITOL-ANCHORED MEMBRANE GLYCOPROTEINS AND PROTEIN TYROSINE KINASES[1]

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A significant fraction of human glycosyl-phospatidylinositol-anchored Ag CD59, CD55, CD48, and CDw52 is present in several cell lines tested (HPB-ALL, Jurkat, HL-60, Raji) in very large noncovalent complexes relatively resistant to dissociation by detergents. These complexes also contain some (glyco)lipids, such as these bearing the CD15, CDw17, and CDw65 determinants, and several intracellular components including protein tyrosine kinases and probably several of their potential substrates. Preclearing of the detergent lysates with different antibodies indicated that all these components are present jointly in a common single type of complexes the size of which is around 100 nm (molecular mass in the range of at least tens of thousands kilodaltons) as determined by ultrafiltration and gel chromatography. These results indicate the existence of cell-surface domains, specifically enriched in the above listed components, that may play a critical role in the so far poorly understood phenomenon of cell activation mediated through many different glycosyl-phospatidylinositol-anchored (glyco)proteins and glycolipids.

Recent studies from our laboratory have shown that the GPI[3]-anchored leukocyte surface gp CD59, CD55, and CD48 on the human HPB-ALL thymoma cell line as well as mouse Thy-1 gp on the EL-4 thymoma are at least partially noncovalently associated with other molecules, such as so far unidentified (glyco)lipids, an 80-kDa gp, and a protein kinase (1). More recently it was found that the association of GPI-anchored (glyco)proteins with PTK is a more general phenomenon and that such complexes can be demonstrated in various cell types (2). These observations may explain the puzzling signal transducing capacity of this class of membrane molecules devoid of any intracellular domains (3, 4). Our initial observations on the existence of these complexes left several questions open, such as: 1) Are there on a single cell type separate complexes involving individual GPI-gp or are the complexes rather uniform with each one containing several different GPI-gp? 2) What is the size and what are other components of these complexes in various cell types? 3) What fraction of GPI-gp and of PTK exist in these complexes as opposed to uncomplexed forms? In this communication we attempt to clarify these points and characterize in more detail this novel type of membrane complexes.

MATERIALS AND METHODS

Reagents and cells. Sepharose 4B and M_r standards were purchased from Pharmacia (Uppsala, Sweden), *N*-bromosuccinimide, autoradiography films, and chemicals for SDS-PAGE from Sigma (St. Louis, MO), NP-40 from Fluka (Buchs, Switzerland), Pansorbin from Calbiochem (La Jolla, CA), Bio-Gel A150m and anti-Ig-peroxidase conjugates from Bio-Rad (Richmond, CA), endoglycosidase F from Lucerna (Luzern, Switzerland), chemiluminescence-enhanced Western blotting kit from Amersham (Aylesbury, UK), [^{125}I]NaI and [γ-^{32}P]ATP from NEN Du Pont (Dreieich, FRG), nitrocellulose for Western blotting and 200- and 50-nm filters from Renner (Dannstadt, FRG), and fluorescent microbeads of defined size Fluoresbrite from Polysciences (Eppelheim, FRG). PI-PLC was kindly provided by Dr. M. G. Low (Columbia University, New York, NY). The HPB-ALL thymoma cell line (CD45$^+$) was originally obtained from the laboratory of Professor J. L. Strominger (Harvard University, Cambridge, MA), myeloid cell line HL-60, B-cell line Raji, T cell lines Jurkat and Molt-4 from Dr. J. Stöckbauer (Institute of Haematology and Blood Transfusion, Praha). The cell lines were grown in RPMI medium supplemented with 10% calf serum.

Antibodies. mAb MEM-43 (IgG2a; CD59), MEM-57 (IgG2a; CD3), MEM-102 (IgG1; CD48), MEM-56 (IgG2b; CD45R), B2M-01 (IgG1a; anti-human β_2-microglobulin), MEM-74 (IgM; CDw17), MEM-59 (IgG1; CD43), MEM-48 (IgG1; CD18) and MEM-118 (IgM; CD55; our unpublished results) were prepared in our laboratory and described elsewhere (5–10). YTH 66.9 (rat IgM; CDw52) (11) was kindly provided by Dr. G. Hale (University of Cambridge, UK), VIMC6 (IgM; CD15) (12) and VIM2 (IgM; CDw65) (13) by Dr. O. Majdić (University of Vienna, Austria), affinity purified goat anti-mouse Ig and goat anti-rabbit Ig were purchased from Sigma, rabbit anti-mouse Ig was prepared and kindly supplied by Dr. L. Slepička (ÚSOL, Olomouc, Czechoslovakia), rabbit antiserum against p56^{lck} (used for Western blotting) was kindly provided by Dr. B. M. Sefton (The Salk Institute, San Diego, CA) (14) and that used for immunoprecipitation by Dr. I. Horak (NIH, Bethesda, MD).

Cell surface radioiodination, immunoprecipitation, lysate preclearing, and in vitro kinase assay. Cell surface radioiodination was performed by a modification of the *N*-bromosuccinimide method (15). Labeled or unlabeled cells ($5–10 \times 10^7$) were lysed for 30 min at 0°C in a lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.2, 2 mM EDTA, 1 mM PMSF, 5 mM iodoacetamide, and 1% NP-40), the insoluble material was removed by centrifugation and the supernatant was used for immunoprecipitation by employing a modification of the solid phase immunoisolation technique (16). Briefly, the wells of flexible polyvinylchloride 96-well microtitration plates (Flow ICN, Meckenheim, FRG) were first coated with anti-mouse (or anti-rabbit) Ig and then with mouse mAb (or rabbit antiserum). Such antibody-coated wells served as immunosorbents for isolation of the respective Ag from detergent lysates. The Ag attached to the immunosorbent wells are further referred to as "immunoprecipitates." In

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[3] Abbreviations used in this paper: GPI, glycosyl-phosphatidylinositol; gp, glycoprotein; PTK, protein tyrosine kinase; NP-40, Nonidet P-40; RaAMIg, rabbit anti-mouse Ig; PI-PLC, phosphatidylinositol-specific phospholipase C.

vitro kinase assays (17) were performed directly in the wells containing the immunoprecipitates obtained from lysates of unlabeled cells using the reaction mixture containing 25 mM HEPES, 5 mM $MnCl_2$, 0.1% NP-40, and 0.1 μCi [γ-^{32}P]ATP in a final volume of 50 μl. After 25 min of incubation at room temperature, the wells were washed with PBS. The immunoprecipitated ^{125}I- or ^{32}P-labeled Ag were finally eluted by sample buffer and analyzed by SDS-PAGE followed by autoradiography. As the capacity of antibody-coated wells was too low, quantitative preclearing of the lysates was performed by means of Pansorbin coated either directly with Protein-A-binding mAb of the IgG2a or IgG2b isotype (MEM-43, MEM-57, MEM-56, B2M-01) or coated first by RaAMIg and then with mouse mAb (IgG1 or IgM). In separate experiments it was verified that the results of preclearing were the same when the IgG2a and IgG2b mAb were adsorbed to Pansorbin directly or indirectly through RaAMIg. Fifty milliliters of packed volume of thoroughly washed antibody-coated Pansorbin were added to 0.5 ml of the detergent lysate and incubated with occasional stirring on ice for 60 min. After 3 min of centrifugation at 13,000 × *g* at 4°C the precleared supernatants were used for further experiments.

Gel chromatography and ultrafiltration. Small columns (0.8 cm × 5 cm, total volume of 3 ml) of Sepharose 4B were washed with 2 volumes of the lysis buffer, 0.3 ml of the cell lysate was applied at the top and left to enter the gel for 5 min; the 0.3 ml of the eluate was collected as fraction 1. Then 0.3 ml of lysis buffer was applied, fraction 2 collected in 5 min, and so on. The fractions were then used for immunoprecipitation or Western blotting. The minicolumns were used in order to minimize the time necessary for separation; in preliminary experiments it was found that the quality of separation was comparable to that obtained with much larger columns (25 ml).

The columns of Bio-Gel A150m (0.45 cm × 16 cm; total volume of 2.5 ml) which was very sensitive to pressure changes were prepared so that a narrow tubing attached to the column outlet was adjusted to collect the fractions 2 cm below the gel level in the column. In this case the samples were 0.1 ml of the lysate containing 0.25% suspension of Fluoresbrite microbeads (of either 466- or 60-nm diameter), and 0.1-ml fractions were collected each 5 min as described above. The fractions were analyzed for the presence of "GPI complexes" by anti-CD59 immunoprecipitation and in vitro kinase assay. The fluorescence of the microbeads was observed in a fluorescent microscope. The maximum concentration of the beads was found in several successive fractions; the maximum was very flat and broad, so that only the range of the most positive fractions could be determined but not a single "maximum fraction." All gel chromatography experiments were performed at 4°C.

The membrane filters of graded pore size were mounted in syringe filter-sterilization adaptors and before being used were washed successively with several milliliters of the lysis buffer containing 1% BSA, then with the cell detergent lysate, and then with the lysis buffer.

Other methods. PI-PLC treatment of cells was performed as previously described (5), SDS-PAGE was performed in the discontinuous system of Laemmli (18), electroblotting was done in a semidry blotting apparatus (Renner), the nitrocellulose replicas were quenched by incubation with 5% defatted dried milk and immunostained by sequential incubation with mAb (ascitic fluid diluted 200×) or polyclonal antiserum (diluted 2000×) followed by an appropriate second antibody conjugated with peroxidase (diluted 10,000×) and chemiluminescence-enhancing substrate solution (Amersham). All antibodies were diluted in PBS containing 0.05% Tween 20 and 1% dried milk. The peroxidase-containing zones were visualized by luminography (1–30 min) on x-ray films. Endoglycosidase F treatment of the immunoprecipitates was performed as described by Štefanová et al. (5).

RESULTS

Several GPI-linked membrane gp, glycolipids, and a PTK are present mostly in a single complex. To determine whether different GPI-anchored surface molecules are complexed separately or jointly to the PTK, we precleared the cell detergent lysates with Pansorbin coated with antibodies against various leukocyte surface molecules and then performed immunoprecipitations followed by in vitro kinase assays. As shown in Figure 1*a*, nearly no kinase activity could be immunoprecipitated by antibodies against any GPI-anchored Ag from the HPB-ALL lysate precleared with an anti-CD59 mAb, whereas preclearing with a control anti-CD3 mAb had no effect. Preclearing with antibodies against the other GPI-gp, CD55, and CD48 also had a similar effect as preclearing with anti-CD59 mAb, whereas preclearing with antibodies against two other control Ag, CD45R and β_2-microglobulin, had no effect (not shown). Similar results were also obtained in cases of two other cell lines, HL-60 and Jurkat (Fig. 1, *b* and *c*): in lysates of these cells antibodies against the GPI-anchored gp CD48 (only in Jurkat), CDw52, CD55, and CD59 and against presumably glycolipid Ag CDw17, CD15, and CDw65 (the latter two only in HL-60) immunoprecipitated a kinase activity, most of which could be specifically removed by preclearing with Pansorbin coated with antibodies against any of these molecules. A notable exception was the CD55 Ag in Jurkat cell lysate: a considerable amount of the kinase activity coprecipitating with this molecule could not be removed by preclearing with Pansorbin coated with the anti-CD59 mAb (see Fig. 3*c*) nor with other relevant antibodies (not shown). This may be due to the presence of the well known alternative transmembrane form of CD55. This point is presently under investigation in our laboratory. These results indicate that several different GPI-gp and glycolipids are noncovalently associated mostly together in a single complex with a protein kinase(s); only a minority of these complexes may lack some of the components. This is also in agreement with previous indications that small amounts of CD55 can be coprecipitated with CD59 (and with glycolipids) and vice versa (1). In the following text we will for the sake of brevity call these particles "GPI complexes."

The possible specific role of the GPI-gp in maintaining the integrity of the GPI complexes was examined by treatment of HL-60 cells with PI-PLC followed by immunoprecipitation and an in vitro kinase assay from the NP-40 lysate of these cells. As shown in Figure 1*d* the PTK-containing complexes could be still precipitated by mAbs against the glycolipid Ag CDw17, CD15, and CDw65, whereas essentially no kinase activity could be immunoprecipitated by mAb against the GPI-gp after this treatment. This is in agreement with the fact that PI-PLC removes most GPI-gp from the HL-60 cell surface (not shown). These results indicate that the extracellular portions of the GPI-gp are not essential to keep the complexes together.

The size and properties of the complexes. Our initial attempts to perform the preclearing experiments described in the previous paragraph by filtering the detergent lysates through columns of Sepharose 4B with immobilized mAb to CD59 reproducibly failed (i.e., an essentially unchanged amount of kinase activity was immunoprecipitated from the lysates passed through the immunosorbent column by anti-CD59, anti-CD55, etc., even though substantial amounts of the CD59 Ag could be released from the immunosorbent by high pH elution). A plausible explanation is that the complexes were too large to enter the pores of the Sepharose 4B gel beads. This explanation was confirmed by the results of gel chromatography on columns of Sepharose 4B (Fig. 2) when nearly all the kinase activity associated with the GPI complexes was present in the fractions of the lysates of HPB-ALL corresponding to the column's void volume. According to the manufacturer's data, the exclusion limit of Sepharose 4B is in the range of tens of millions of daltons. Essentially the same results were obtained when

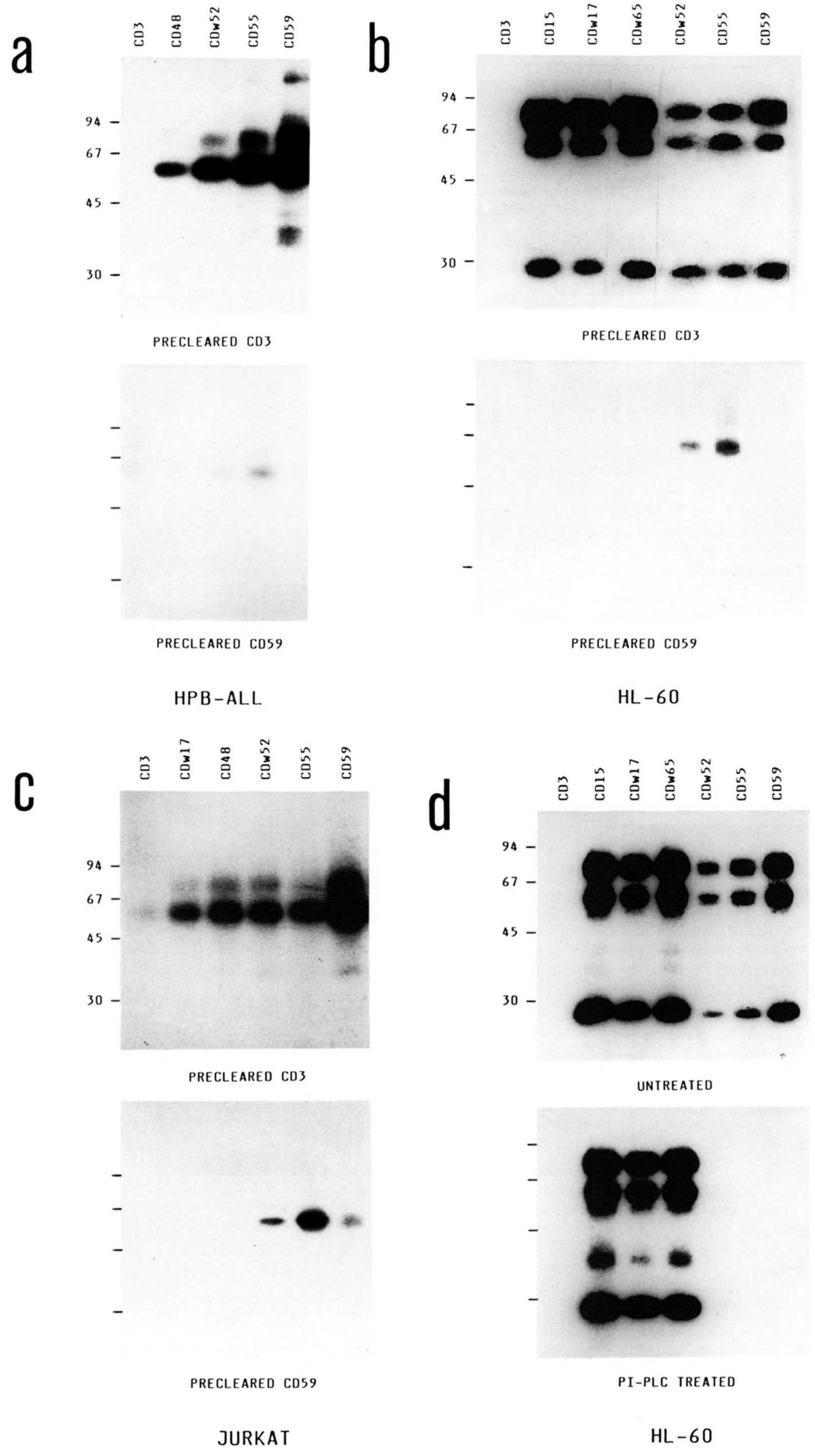

Figure 1. Removal of GPI-gp-associated protein kinase activity by preclearing with mAb against various GPI-gp. *a–c*, The lysates of the indicated cell lines were precleared with Pansorbin coated either with the anti-CD3 mAb MEM-57 (negative control) or anti-CD59 mAb MEM-43 as described in *Materials and Methods*, and then immunoprecipitation with mAb against the indicated Ag, in vitro kinase assay, SDS-PAGE, and autoradiography were performed. A substantial drop in the phosphorylation intensity similar to that shown in the *lower panels* (preclearing with anti-CD59) was observed after preclearing with the other mAb against the GPI-gp (not shown), whereas similar results (*upper panels*) were observed without any preclearing and also after preclearing with anti-CD45R or anti-β_2-microglobulin (not shown). Preclearing with the anti-CD3 mAb-coated Pansorbin completely removed the CD3 Ag (not shown). *d*, The effect of the PI-PLC treatment of HL-60 cells on the GPI complexes. *Upper pannel*, the results of in vitro kinase assays of the indicated immunoprecipitates from the untreated cells; *lower panel*, the same from PI-PLC-treated cells. Positions of M_r standards (values given only in the *upper panel*) are shown at the *left margin*.

detergent lysates of other cell lines (Jurkat, Molt-4, HL-60, Raji) were subjected to gel chromatography on Sepharose 4B, immunoprecipitation with anti-CD59 (or antibodies against the other GPI-gp) and in vitro kinase assay: in all cases the kinase activity was nearly exclusively found in the void volume fraction (not shown).

To check semiquantitatively what part of the total CD59 is present in the GPI complexes, the fractions obtained after gel chromatography were subjected to SDS-PAGE and Western blotting. As shown in Figure 3, CD59 molecules were present in comparable amounts in two fractions. The first comigrated with the peak of

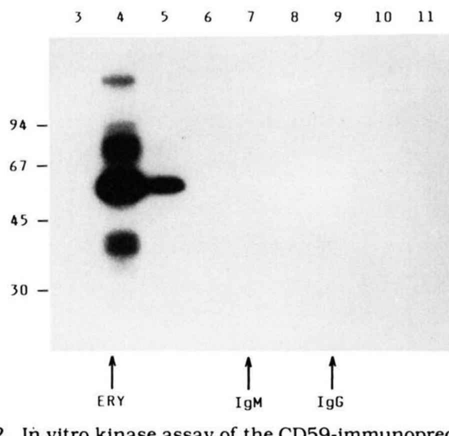

Figure 2. In vitro kinase assay of the CD59-immunoprecipitates from Sepharose 4B gel chromatography fractions of HPB-ALL lysate. All details on gel filtration are given in *Materials and Methods*. Fraction numbers (each 0.3 ml) are given at the *top*, and positions of M_r standards (see Fig. 1) at the *left margin*. Elution volumes (maximum concentration) of E (*ERY*), IgM, and IgG are shown by *arrows* at the *bottom*.

kinase activity (void volume) and the second corresponded to the elution volume of relatively small molecules. In contrast, another abundant leukocyte gp CD43 was absent from the void volume fraction (Fig. 3*a*), as well as CD3, β_2-microglobulin, and CD18 (data from HPB-ALL, not shown). Two other GPI-anchored Ag, CD55 (Fig. 3*c*) and CD48 (not shown), were found mainly in the void volume fraction. The PTK p56^{lck} which was previously shown to be associated with GPI complexes (2) had a distribution qualitatively similar to CD59 (Fig. 3*d*). It should be noted that the p56^{lck} observed in the void volume fraction consisted exclusively of the form with the slightly higher apparent M_r.

The "void volume-fraction" of CD59 and p56^{lck} essentially disappeared from the HPB-ALL lysates after preclearing with an anti-CD55 mAb adsorbed to Pansorbin, whereas the presence of these molecules in the "low M_r" fractions was unaffected after this treatment (Fig. 4). The kinase activity precipitated by antibodies against p56^{lck} also nearly completely disappeared after preclearing the lysate with Pansorbin coated with the mAb to CD59 from the void volume fraction but not from the low M_r fractions (Fig. 5). This again confirms that in the GPI complexes several GPI-gp are present mostly jointly and, importantly, that the other, low M_r fraction of CD59 is not associated with either the kinase or with CD55 (and presumably neither with other GPI-anchored molecules). The p56^{lck} associated with the GPI complexes seems to represent only a minor (but disproportionately more active; Fig. 5) fraction of total p56^{lck} and it is exclusively the form corresponding to the upper band of the doublet.

The amount of CD59 (or other GPI-gp) estimated by Western blotting in the gel chromatography fractions represents of course both the cell surface and any intracellular Ag. To get more specific information on the distribution of surface-expressed CD59, the detergent lysate of surface radioiodinated HPB-ALL cells was subjected to gel chromatography on Sepharose 4B column followed by immunoprecipitation from the fractions. In agreement with our previously published data (1), the anti-CD59 mAb coprecipitated several other components (e.g., a prominent 80-kDa zone and a low M_r material corresponding to glycolipids) from the unseparated lysate. These components were observed only in the CD59 immunoprecipitates obtained from the void volume fractions, whereas in the case of the low M_r fractions, the CD59 Ag was immunoprecipitated apparently without any associated components (Fig. 6). Similarly, as observed in the previously shown results of Western blotting (Fig. 3), this experiment also confirmed that a sizeable fraction (approximaterly 20% as judged from quantitative measurement of radioactivity of the CD59 (18-kDa) zone) of the surface CD59 is present in the large

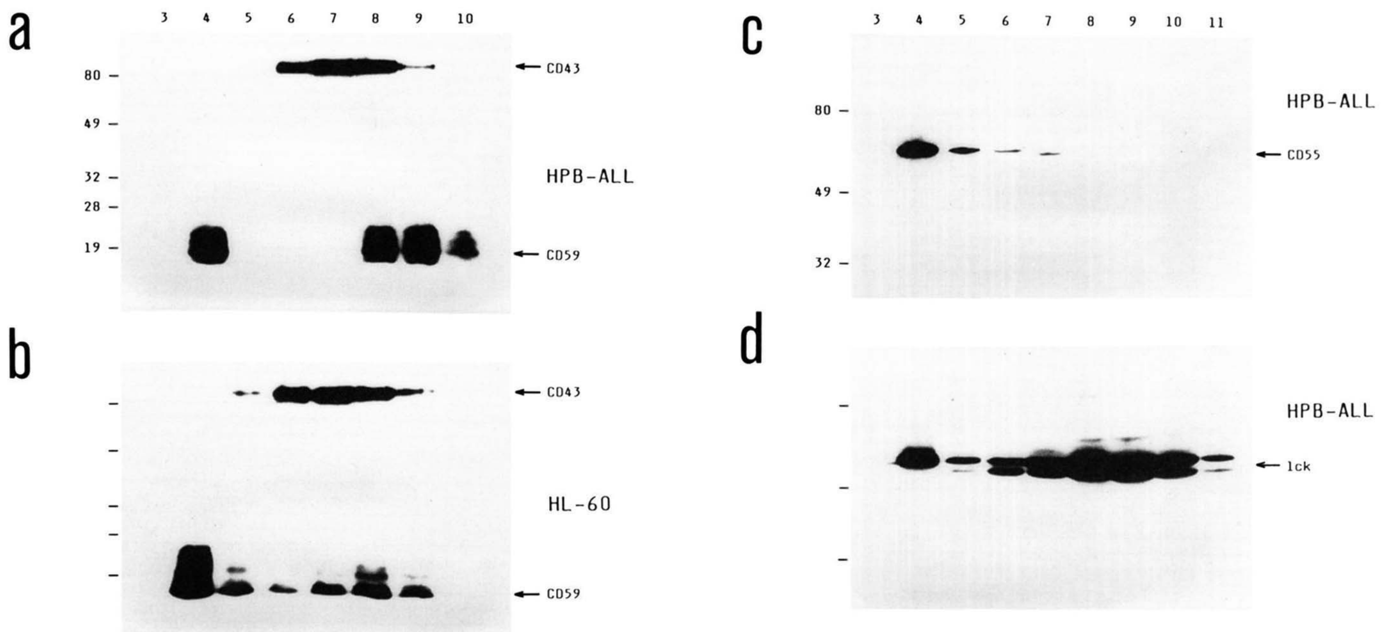

Figure 3. Western blotting from the Sepharose 4B gel chromatography fractions. The antibodies and conditions used are given in *Materials and Methods*. The *blots a* and *b* were stained with a mixture of anti-CD59 and anti-CD43 mAb; each antibody reacted only with the appropriate Ag, as ascertained separately (not shown). The *blots c* and *d* were stained only with the appropriate antibodies (anti-CD55 and anti-p56^{lck}, respectively). Positions of the respective molecules, the cells used as well as positions of M_r standards (see Fig. 1), and numbers of the gel chromatography fractions are indicated. The negative results of staining with irrelevant negative controls are not shown.

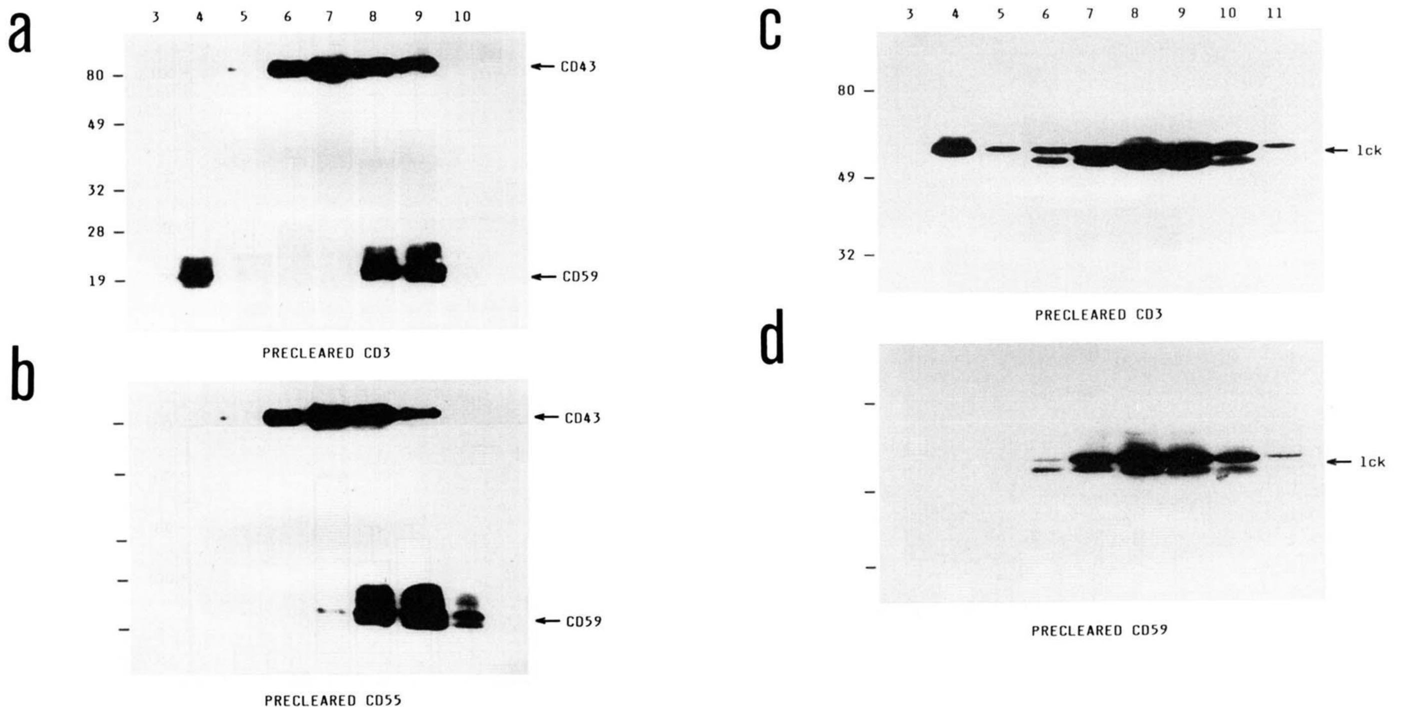

Figure 4. Western blotting from the Sepharose 4B gel chromatography fractions of the HPB-ALL lysates precleared with anti-CD3 mAb (negative control) (*a* and *c*), anti-CD55 (*b*), or anti-CD59 (*d*). All other details as in Figure 3.

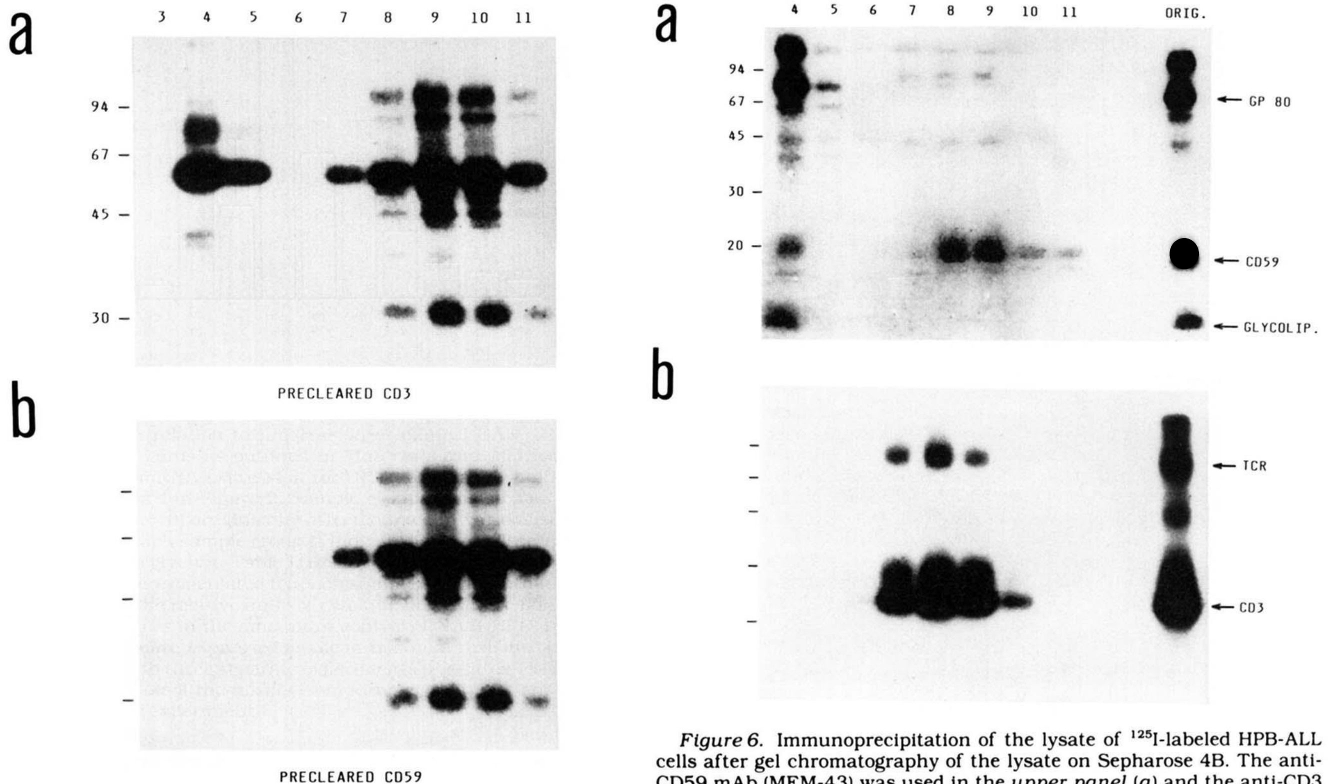

Figure 5. In vitro kinase assay of the $p56^{lck}$ immunoprecipitates from Sepharose 4B gel chromatography fractions; the samples applied on the columns were the lysates precleared with Pansorbin coated with anti-CD3 (negative control (*part a*)) or with anti-CD59 (*part b*). For other details see Figure 2.

Figure 6. Immunoprecipitation of the lysate of ^{125}I-labeled HPB-ALL cells after gel chromatography of the lysate on Sepharose 4B. The anti-CD59 mAb (MEM-43) was used in the *upper panel* (*a*) and the anti-CD3 mAb (MEM-57) in the *lower panel* (*b*). Nonreduced samples were analyzed on 12.5% gels. The positions of the respective molecules are indicated. The *far-right lane* (*ORIG.*) represents the samples precipitated from the original lysate before gel chromatography. The numbers of fractions and positions of M_r standards are indicated.

complexes, whereas the rest is apparently uncomplexed. As expected, CD3 tested as a control (and also CD43 and CD18; not shown) could be immunoprecipitated only from the low M_r fractions.

To determine more exactly the size of the GPI complexes, the detergent lysate of HPB-ALL cells was filtered through membrane filters of graded pore sizes. The kinase activity coprecipitated with CD59 was completely

lost after passing the lysate through the 50-nm filter, whereas no change was observed after filtration through a 200-nm filter (Fig. 7*a*). The filtered lysates were also subjected to gel chromatography on Sepharose 4B followed by Western blotting. As expected, the profile of the fractions from the 200-nm filtrates was nearly the same as before any filtration, whereas after filtration through the 50-nm filter the CD59 present in the void volume fraction was completely lost. The other, low M_r fraction of CD59 was unchanged; similarly unchanged was the amount and distribution of the control Ag CD43 (Fig. 7*b*).

Finally, the HPB-ALL lysate was filtered through a column of an extremely porous gel Bio-Gel A150m and the position of the peak of kinase activity that coprecipitated with CD59 was compared with elution volumes of the Fluoresbrite microbead size standards. According to these results, the size of the GPI complexes appears to be larger than 60 nm (Fig. 8). The next larger beads available (150 nm) had elution volume indistinguishable from the peak of the GPI complexes (and the 466-nm beads) thus indicating that all these particles were too large to enter the gel pores, and so a more exact determination could not be achieved by this method (T. Cinek, unpublished data).

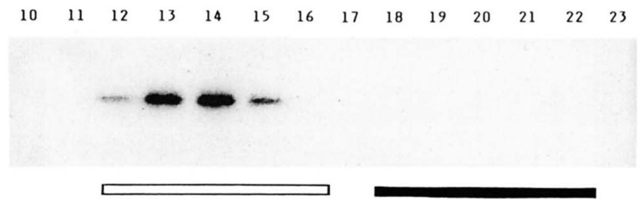

Figure 8. Comparison of gel chromatography behavior of the GPI complexes and size standards (Fluoresbrite microbeads) in Bio-Gel 150m (performed as described in *Materials and Methods*). The protein kinase activities of the CD59 immunoprecipitates in the column fractions (0.1 ml; numbers of fractions are given at the *top*) are shown (only the relevant part of the gel). The fractions containing maximum concentrations of the 466- and 60-nm microbeads are marked by the *open* and *closed bar*, respectively.

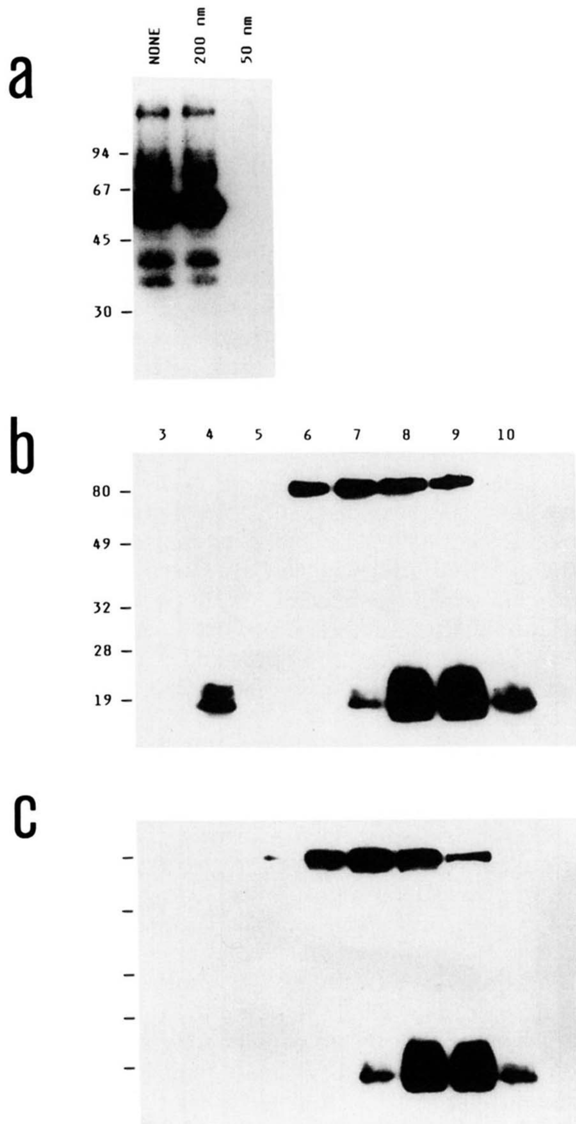

Figure 7. The size of GPI complexes as determined by ultrafiltration. *a*, The results of the in vitro kinase assay after immunoprecipitation on the anti-CD59 mAb MEM-43 from nonfiltered and 200-nm and 50-nm filtered HPB-ALL lysates. *b*, Western blotting from Sepharose 4B gel chromatography fractions of the lysates filtered through a 200-nm or 50-nm filter, respectively. All details as in Figures 3 or 4.

DISCUSSION

The results of the present study demonstrate that at least a part of the GPI-anchored gp of several human leukocyte cell lines are present in very large noncovalent complexes relatively resistant to dissociation by the commonly used nonionic detergent NP-40. These complexes contain several different GPI-anchored gp, some glycolipids and probably several intracellular proteins, including PTK (which was identified as the $p56^{lck}$ in the case of T cells (2)). The nature of the other components that can be phosporylated in the immunoprecipitates (i.e., the 80-kDa component present in all immunoprecipitates, the weak 35–40-kDa and marked 25-kDa components observed in the HPB-ALL and HL-60 immunoprecipitates, respectively) is presently unknown. The intensity of these additional phosphorylated zones varied somewhat in different experiments. It seems likely that these molecules are potential substrates of the PTK associated with the GPI complexes.

The prominent 80-kDa surface gp associated with the GPI complexes in HPB-ALL cells (Fig. 6) was present only in the "GPI-Ag" immunoprecipitates from HPB-ALL cells (I. Štefanová, unpublished results), which may suggest that it is a molecule of very restricted expression without a more general role in the organization of the GPI complexes. It can be speculated that it is just another GPI-anchored surface gp of the HPB-ALL cells, but detailed characterization of this component will be possible only after obtaining a specific mAb. In our previous paper (1) we suggested that this gp could be identical to the 80-kDa component that is phosphorylated in vitro in the "GPI-gp" immunoprecipitates. This suggestion seems to be very unlikely because the 80-kDa phosphorylated protein is present even in precipitates from the cells in which no ^{125}I-labeled GPI-gp-associated 80-kDa gp could be found, and moreover, the phosphorylated protein is, in contrast to the ^{125}I-labeled gp in HPB-ALL, insensitive to endoglycosidase F (T. Cinek, unpublished observation).

It should also be pointed out that in the present paper we demonstrated for the first time coprecipitation of protein kinase activity with one additional GPI-anchored gp (CDw52) (19), and with three presumably glycolipidic Ag CD15 (CD15 determinants are actually known to be present both on several gp and glycolipids (20)), CDw17

(21), and CDw65 (22). This gives further support to the idea that this phenomenon is rather general as was already indicated by our previous results (1, 2). As shown in the present paper, the kinases are associated only with the large GPI complexes but not with the low M_r forms of the GPI-anchored Ag. It is not clear how closely our in vitro experiments reflect the in vivo situation; it is possible that during solubilization some loosely bound components of the complexes are lost or that some complexes more or less dissociate giving rise to the observed "uncomplexed" CD59. On the other hand, the GPI complexes appear to be rather stable even under more stringent conditions than those used routinely in the present study. They could be demonstrated after gel chromatography by Western blotting even if the lysis buffer in addition to 1% NP-40 also contained 1% sodium deoxycholate and 0.1% SDS (T. Cinek, unpublished results). It is of interest that removal of the extracellular portions of the GPI-gp by PI-PLC does not affect the integrity of the complexes (Fig. 1*d*). Therefore, the lipid moieties and their interactions within the membrane may be most important. Nevertheless, a specific critical role of some specific glycolipid- or PI-PLC-resistant protein cannot be ruled out. The present biochemical data remain to be corroborated, e.g., by comodulation experiments on living cells, but it should be noted that tyrosine phosphorylation immediately follows GPI-gp cross-linking (2, 23), which suggests that the association with PTK does exist in vivo.

In the present study we have not investigated the possible relationship between the GPI complexes and the well known complexes of CD4, CD8, and TCR/CD3 with PTK $p56^{lck}$ and $p59^{fyn}$, respectively, as the conditions used (presence of iodoacetamide in the lysis buffer in the case of CD4 and CD8 association with $p56^{lck}$ and the detergent NP-40 in the case of TCR/CD3 association with $p59^{(fyn)}$) are known to interfere with the stability of such complexes (24, 25). The presence of iodoacetamide in the lysis buffer, as already noted (2), considerably enhanced the reproducibility and intensity of the in vitro phosphorylation in GPI-gp immunoprecipitates which might be due to inhibition of some critical phosphatases or proteases in the lysate. However, comparative studies on the properties (size, composition, and possible relationship to the GPI complexes under some conditions) of the CD4-, CD8-, and TCR/CD3-PTK complexes are now in progress in our laboratory.

An interesting aspect of the present study is that the GPI complexes contain only a minor fraction of total $p56^{lck}$, but this fraction appears to have disproportionately high self-phosporylating activity (cf. Figs. 4 and 5). The $p56^{lck}$ is present in the HPB-ALL lysate as two species of similar M_r; the GPI complexes contain selectively only the one with higher M_r. It should be also noted that the profile of other in vitro phosphorylated zones in the $p56^{lck}$ precipitates is different in the GPI complexes (void volume fractions) and in the low M_r fractions not associated with GPI-gp. This indicates a specificity of associations with other components within the GPI complexes.

So far the existence of the very large complex fraction seems to be unique for GPI-anchored surface gp, as none of the tested control transmembrane Ag was detected in the void volume fractions of the Sepharose 4B columns. Our results also indicate that the method used for immunoisolation can profoundly affect the results—thus, the commonly used agarose gel-based immunosorbents may be unsuitable in cases of Ag present in very large complexes.

We suggest that our observations reflect the existence of membrane domains specifically enriched in GPI-anchored (glyco)proteins and (glyco)lipids associated with intracellular components linked to the membrane presumably also by hydrophobic moieties such as myristoyl residues. Such complexes could of course also contain certain so far undetected "conventional" (transmembrane-anchored) gp, but our preclearing data show that at least some abundant transmembrane proteins such as CD3, CD45, and class I MHC gp are absent from these "domains." It should be noted that a number of mAb against various other non-GPI-gp failed to coprecipitate any kinase activity under the conditions used here for solubilization (1, 2), thereby suggesting that these were not present in the GPI complexes. Only rigorous purification of the GPI complexes from different cell types and detailed analysis of their components will provide the ultimate information in this respect. It is easy to imagine that cross-linking of the components within such a domain (or between domains) by means of suitable antibodies may induce changes in the intracellular components of these domains such as protein kinases resulting in protein phosphorylation and cell activation. This could be the structural basis for the strikingly frequently observed signal transducing potential of GPI-anchored (glyco)proteins (3, 4) and glycolipids (26). The existence of possibly similar lipid membrane domains with specifically segregated components has been described previously (27, 28). Potentially relevant to our data is the observation that the GPI-anchored 5-tetrahydrofolate receptor exists in the membrane of kidney cells in large clusters, apparently stabilized by cholesterol. The clusters are easily observable by immunofluorescence microscopy (29). It is not known whether these clusters also contain other GPI-anchored (glyco)proteins and glycolipids. So far we have not been able to observe any clearly clustered distribution of the GPI-anchored Ag by immunofluorescence microscopy. This might be because of the smaller size of these clusters. Nevertheless, one would expect that electron microscopy should be a suitable technique to detect complexes of this size in intact cell membranes.

It is not clear at present what might be a physiologic role of these GPI complexes. A number of GPI-anchored gp have well known functions presumably requiring coupling to signal-transducing machinery. The monocyte-specific gp CD14 is the receptor for the LPS-LPS-binding protein complex (30), and possibly also an adhesion molecule (31). CD58 (32), CD66 and CD67 (33), and probably also CD48 (34) and a mouse T cell Ag Thy-1 (35) are also cell adhesion molecules (but an alternative transmembrane form of CD58 exists as well). GPI-anchored forms of two other adhesion molecules, a fibronection receptor (36) and LAM-1 (37), may also exist. The GPI-anchored form of CD16 is a low affinity receptor for IgG in neutrophils (38), whereas CD55 and CD59 are C-protecting molecules (39, 40). Another GPI-anchored molecule, CD73 is an ectoenzyme (5′-nucleotidase) (41) and possibly also an adhesion molecule (42). Several other GPI-anchored cell surface ectoenzymes such as acetylcholinesterase and alkaline phosphatase also exist (43),

and it is possible that their interaction with substrates may provide signals to the cells carrying them. The receptor for the plasminogen activator structurally similar to CD59 and murine Ly6 also belongs to this class of membrane proteins (44). The roles of several other GPI-anchored leukocyte surface molecules such as CD24 (45), CDw52 (19), prion protein precursor (46), murine J11d (47), Qa-2 (48), and Ly-6 (49), and rat RT6 (50) are not known.

An alternative possibility is that the "GPI domains" are, e.g., precursors of exocytic vesicles that are shed from a variety of cells (51–53). The vesicles shed from E after induction by heating or by ATP depletion are specifically enriched in at least two GPI-anchored proteins, acetylcholinesterase and CD55 (54). The exocytosis of similar membrane vesicles appears to be an important mechanism through which cells get rid of the membrane attack complex of the C (55); the fact that CD59 is a C-protecting molecule interacting with C8 and C9 (40, 56) may be relevant in this respect, and the GPI domains could play a role in more efficient protection from the attack by homologous C either by providing a higher local concentration of the complement protecting molecules and/or by serving as easily removable membrane regions upon damage. However, it is possible that an entirely different function of these GPI-complexes/domains will be found after their purification to homogeneity from various types of cells and detailed analysis of all their components. Another question emerging from these studies is whether other large complexes of size comparable to the GPI complexes exist that involve other transmembrane-anchored membrane components, such as well known receptors, membrane enzymes, etc.

After finishing this work we became aware of a recent paper (57) describing the existence of very large detergent-resistant membrane domains of epithelial cells specifically enriched in GPI-gp and glycolipids. These data further support our present conclusions and indicate that the existence of the GPI complexes may be a rather general phenomenon occurring in various types of cells.

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