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ASSOCIATION OF THE CD59 AND CD55 CELL SURFACE GLYCOPROTEINS WITH OTHER MEMBRANE MOLECULES

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mAb against human glycosyl-phosphatidylinositol-linked leucocyte surface Ag CD59 and CD55 immunoprecipitated from detergent lysates of HPB ALL cell line in addition to the respective Ag a common 80-kDa glycoprotein component and (glyco)lipids. The 80-kDa glycoprotein is different from otherwise similar CD44 Ag. The CD59 immunoprecipitate contained also a small amount of the CD55 glycoprotein and the CD55 immunoprecipitate minute amount of the CD59 Ag. These results are interpreted in terms of existence of noncovalent complexes resistant to dissociation by mild detergents and consisting of the 80-kDa glycoprotein, CD59 and CD55 glycoproteins, relatively tightly bound (glyco)lipids and possibly other so far unidentified components. These complexes contain probably also other glycosyl-phosphatidylinositol-linked Ag, as an anti-CD48 mAb immunoprecipitated also an apparently very similar complex. The complexes immunoprecipitated by mAb against the CD55, CD59, and CD48 Ag also contain a protein kinase activity. This type of complexes could not be demonstrated in several other cell types such as RBC, PBMC, and HeLa cells. However, a qualitatively very similar set of components was immunoprecipitated from the murine thymoma EL-4 cell line by an anti-Thy-1 mAb.

Recently we have described an 18- to 20-kDa broadly expressed GPI[2]-anchored human cell surface gp similar to murine Ly-6 Ag (1) that was later given the name CD59 (2). Complementary DNA coding for the CD59 polypeptide were cloned in several laboratories (3–6). The CD59 gp (described in literature under several names) plays an essential role in protection of cells from autologous complement lysis (3, 7, 8) and may be equally important in regulation of platelet activation by complement (9). In addition, it may be involved also in T cell adhesion and activation (10). In our initial work we observed an unidentified 80-kDa component noncovalently associated with the 18- to 20-kDa CD59 Ag (1). In the course of our attempts to elucidate the nature of the 80-kDa component as presented in this report we found that at least a fraction of the CD59 molecules are tightly associated in addition to the 80-kDa glycoprotein with membrane (glyco)lipids and a protein kinase activity. Subsequently, we found that very similar complexes were immunoprecipitated with mAb against two other human GPI-linked membrane Ag, CD55 and CD48 that are strongly expressed on the HPB ALL cell line used predominantly in our experiments. CD55 is identical to the broadly expressed, structurally and functionally well defined complement regulatory protein decay-accelerating factor (11). Biologic role of the pan-leucocyte gp CD48 is not known but amino acid sequence of its polypeptide chain derived from recently cloned cDNA (12, 13) is similar to the adhesion molecule LFA-3 (CD58).

Our present data indicate that at least in some cell types several and perhaps most of the GPI-linked membrane gp are associated with other cell-surface components, which may be relevant to, for example, the well known but unexplained cell activation through many GPI-linked surface molecules (14).

MATERIALS AND METHODS

Reagents and cells. The reagents used and their sources were as follows: CNBr-Sepharose 4B and M_r standards (Pharmacia, Uppsala, Sweden); PMSF, neuraminidase, V8 protease, and chemicals for PAGE (Serva, Heidelberg, FRG); N-bromosuccinimide, NP-40, aminoethylcarbazole, and affinity purified goat anti-mouse Ig antibodies (Sigma Chemical Co., St. Louis, MO); endoglycosidase F (Boehringer, Mannheim, FRG); nitrocellulose membrane (Schleicher & Schüll, Dassel, FRG); ^{125}I-NaI and [γ-^{32}P]ATP (Amersham, Aylesbury, UK); swine anti-mouse Ig-horseradish peroxidase conjugate (Sevac, Praha, Czechoslovakia). PBMC were obtained from the blood of healthy volunteers by a modification of the method by Bøyum (15). The HPB ALL cell line was originally obtained from the laboratory of Professor J. L. Strominger (Harvard University, Cambridge, MA), HeLa cell line was provided by Dr. V. Viklický (Institute of Molecular Genetics, Praha, Czechoslovakia), and EL-4 cell line by Dr. J. Bubeník (Institute of Molecular Genetics, Praha, Czechoslovakia). The cell lines were grown in RPMI medium supplemented with 10% calf serum.

Antibodies. mAb MEM-43 (CD59), MEM-57 (CD3), MEM-31 (CD8), MEM-75 (CD71), MEM-28 (CD45), MEM-48 (CD18), MEM-85 (CD44), MEM-59 (CD43), and B2M-01 (anti-human b2m) were described elsewhere (1, 16–20), mAb K20 (CD29) (21) was kindly provided by Dr. A. Bernard (Institut Gustave Roussy, Villejuif, France), RFT2 (CD7) (22) by Dr. G. Janossy (Royal Free Hospital, London, UK), IA10 (CD55) (23) by Dr. M. B. Whitlow (New York University Medical Center, New York, NY), 1aG4 directed against the murine Thy-1.2 Ag (24) by Dr. P. Dráber (Institute of Molecular Genetics, Praha, Czechoslovakia), mAb WM68 (CD48) originating from the laboratory of Dr. A. J. Henniker (Westmead Hospital, Westmead, Australia) was obtained as a part of the nonlineage/NK panel of the Fourth International Workshop on Human Leucocyte Differentiation Antigens (25).

Cell surface radioiodination, immunoprecipitation, and immunoaffinity chromatography. Cell surface radioiodination was performed by a modification of the previously described method for radioiodination of proteins using N-bromosuccinimide as oxidizing agent (26). HPB-ALL, HeLa cells, PBMC, or RBC (2×10^7) were incubated 2 min at 0°C in 400 μl of PBS in the presence of 1 mCi ^{125}I-NaI and 5 μg N-bromosuccinimide. After washing in ice-cold

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[2] Abbreviations used in this paper: GPI, glycosyl-phosphatidylinositol; gp, glycoprotein; NP-40, Nonidet P-40.

PBS, the cells were lyzed 30 min at 0°C in a 1% NP-40-containing isotonic lysis buffer (140 mM NaCl, 10 mM Tris-HCl pH8.2, 2 mM EDTA, 1 mM PMSF, 1 mM iodoacetate, and 1% NP-40), insoluble components were removed by centrifugation (either 10,000 × *g*, 15 min, or 100,000 × *g*, 60 min, with similar results of subsequent immunoprecipitation) and the supernatant (fresh or frozen, with virtually identical results) was used for immunoprecipitation using the solid phase immunoisolation technique (27). It should be noted that in this technique the wells of plastic microculture plates are first coated with anti-mouse Ig antibodies (in our case affinity purified goat anti-mouse Ig) then with the mouse mAb and such wells serve as immunosorbents for isolation of the respective radiolabeled Ag. The antigens attached to the immunosorbent wells are further referred to as "immunoprecipitates"; lipid extraction and phosphorylation of these immunoprecipitates described below were thus performed directly in the immunosorbent wells. The unlabeled CD59 Ag was purified from HPB-ALL and HeLa cells solubilized in the lysis buffer containing 1% NP-40 (performed in the same way as described above for immunoprecipitation) by affinity chromatography on a column of the MEM-43 mAb immobilized on CNBr-Sepharose 4B as described before (1) and the preparation was further analyzed by Western blotting.

Methods for biochemical characterization of isolated antigens. Deglycosylation of the immunoprecipitated Ag, Western blotting, and SDS-PAGE were performed by modified standard methods as described in more detail before (1). V8 protease treatment was done as follows: the 80-kDa component coprecipitating with the CD55 and CD59 Ag or the band of the CD44 Ag immunoprecipitated from the surface labeled RBC were localized in the unfixed gel by brief autoradiography, the corresponding zones were cut out, finely homogenized, and the radiolabeled Ag was eluted by an overnight incubation at 37°C with a 10-fold volume of a solution containing 0.1 M NH_4HCO_3, 0.1% SDS, 1 mM PMSF, and 5 mM iodoacetate. After addition of 10 μg BSA the supernatant was lyophilized, SDS extracted with methanol, and this purified Ag was dissolved in 40 μl of 0.1 M Tris-HCl buffer pH 6.8 and incubated for 1 h at 37°C with 0.5 μg of V8 protease. The sample was then diluted 1:1 with 2× concentrated sample buffer and analyzed by SDS-PAGE followed by autoradiography.

TLC of (glyco)lipids. The radiolabeled (glyco)lipids were extracted from the immunoprecipitates by a chloroform/methanol mixture (2:1) and then separated on the high-performance TLC plates (Kieselgel G60, Merck, Darmstadt, FRG) using the chloroform/methanol/water mixture (120:70:17, v/v/v) containing 0.02% $CaCl_2$ as the mobile phase, and detected by autoradiography.

Protein kinase activity assay. Solid-phase immunoisolation was performed as described above for immunoprecipitation of radioiodinated antigens, except that lysates of unlabeled HPB ALL cells were used. The reaction mixture 20 mM HEPES, 10 mM $MnCl_2$, and 1 μCi [γ-^{32}P]ATP (28) in a final volume of 50 μl was added to the "immunoprecipitates" isolated in the antibody-coated wells. After incubation for 5 min at 30°C the wells were washed with 1% NP-40-containing lysis buffer, the Ag were then eluted with the sample buffer and analyzed by SDS-PAGE followed by autoradiography.

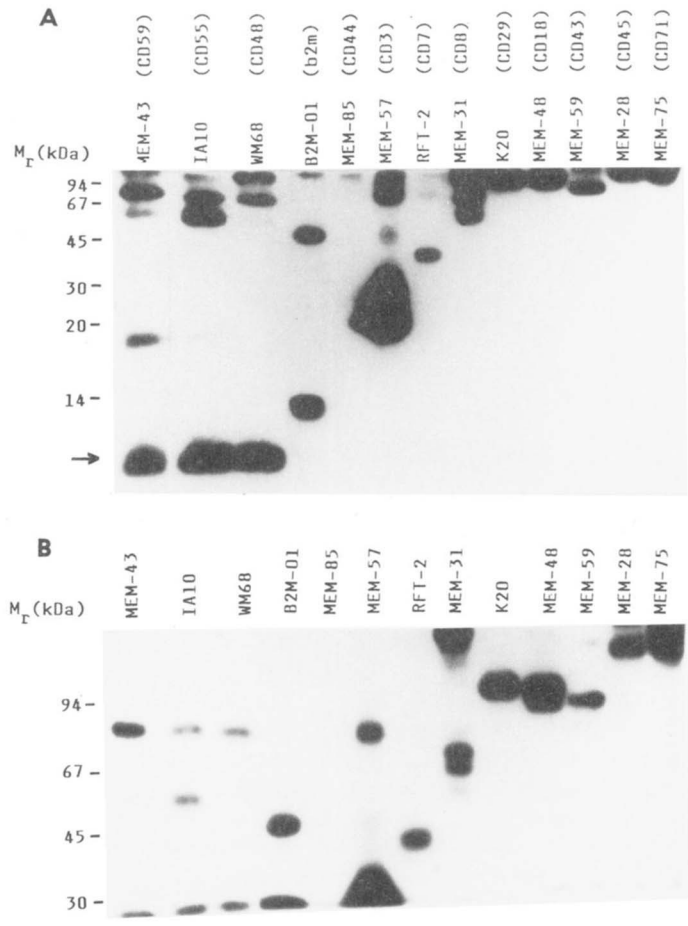

Figure 1. SDS-PAGE and autoradiography of nonreduced samples of the Ag immunoprecipitated from the detergent lysate of ^{125}I-labeled HPB ALL cells by the indicated mAb. In *A*, the 15% gel resolves optimally the low M_r components including the presumed (glyco)lipids migrating with the dye front (*arrow*). *B* (7.5% gel) resolves optimally the components of higher M_r and shows that the mobility of gp80 (in the CD59, CD55, and CD48 immunoprecipitates) is different from e.g., CD29, CD18, or CD43. The absence of the second zone (corresponding to the CD11a chain of LFA-1) in the CD18 immunoprecipitate is due to the lability of the complex upon freezing/thawing of the lysate (I. Štefanová, unpublished observations). Apparent M_r of some components (e.g., CD55) is lower than the real value because nonreduced samples were analyzed (see also the difference of reduced vs nonreduced samples in Fig. 2).

RESULTS

Coprecipitation of additional components with CD59 and CD55 Ag. As observed before (1), the anti-CD59 mAb MEM-43 immunoprecipitated from the detergent lysate of surface radioiodinated HPB ALL thymoma cell line in addition to the 18 to 20 kDa CD59 molecules also an 80-kDa component; in addition, another ~55-kDa zone and a strong zone corresponding apparently to a low M_r substance were observed on the autoradiograms of the immunoprecipitated samples subjected to SDS-PAGE under nonreducing conditions (Fig. 1); qualitatively very similar pattern was observed in the case of reduced samples (see below). These additional components were not immunoprecipitated by mAbs against several other surface antigens of the HPB ALL cells, but a similar set of components was observed in the material immunoisolated by means of antibodies against two other GPI-linked Ag, CD55 and CD48 (Fig. 1). Essentially identical results were obtained when other mild detergents such as CHAPS or β-octylglucoside were used for HPB ALL solubilization instead of NP-40 or when EDTA was omitted from the lysis buffer (not shown). These additional components were not immunoprecipitated by anti-CD59 and anti-CD55 mAb from several other cell types, such as RBC, HeLa, and PBMC (not shown).

Identity of components of complexes associated with CD59 and CD55 glycoproteins. The 80-kDa components coprecipitated with CD59 and CD55 behaved virtually identically with respect of the effects of reduction, neuraminidase and endoglycosidase F treatments (Fig. 2): the difference in mobility of the reduced vs nonreduced samples indicate the presence of intrachain cystine bridges, the effects endoglycosidase F and neuraminidase suggest the presence of an ~5 to 10 kDa N-glycosidically bound carbohydrate moiety of the complex type and limited sialylation of the glycoprotein. The lower intensity of the 80-kDa zone and the presence of a high M_r material under nonreducing conditions may indicate that the gp80 may partially exist as covalently linked oligomers (possibly arising artificially under the conditions of SDS-PAGE). The patterns of the zones obtained after V8 protease treatment were also identical (Fig. 3). The 55-kDa component observed in the CD59 immunoprecipitate behaved

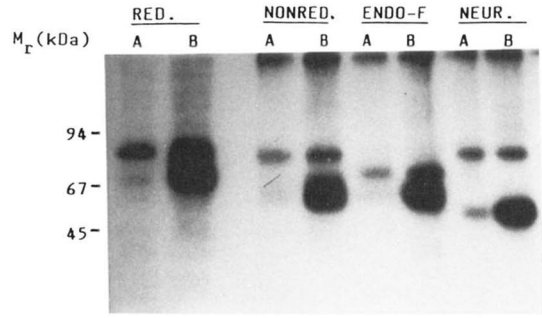

Figure 2. SDS-PAGE (10% gel) and autoradiography of the immunoprecipitate obtained from the detergent lysate of ^{125}I-labeled HPB ALL cells by mAb MEM-43 (CD59) and IA10 (CD55) (*A*, CD59; *B*, CD55). Comparison of mobilities of reduced, nonreduced, endoglycosidase-F- and neuraminidase-treated samples. The enzyme-treated samples were analyzed as nonreduced. Only the relevant part of the gel is shown; the CD59 glycoprotein migrating here with dye front is therefore absent. It should be noted that the clear change of CD55 zone mobility after neuraminidase treatment indicates that the enzyme was active under the conditions used.

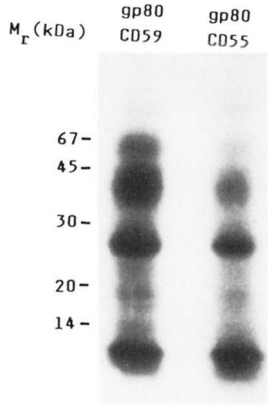

Figure 3. SDS-PAGE (15% gel) and autoradiography of V8 protease digests of gp80 originating from the CD59 and CD55 immunoprecipitates (see *Materials and Methods*).

identically to the major 55-kDa zone in the CD55 immunoprecipitate (Fig. 2). In addition, the 55-kDa zone present in the material isolated on the MEM-43 (anti-CD59) immunosorbent was identified as the CD55 Ag by means of Western blotting (Fig. 4). However, the CD55 immunoprecipitate contained a small amount of a material of M_r corresponding to the CD59 Ag (poorly visible in Fig. 1 but clearly discernible on overexposed autoradiograms). It should be noted that the material isolated by immunoaffinity chromatography on the anti-CD59 mAb MEM-43 immunosorbent from HeLa cells (very rich in both CD59 and CD55 Ag) did not contain any detectable CD55 Ag in agreement with the results of immunoprecipitation, which rules out the possibility of a "nonspecific" contamination or antibody cross-reactivity. Therefore, it is concluded that the 80-kDa components noncovalently associated with the CD55 and CD59 Ag are identical and that these two Ag are partially noncovalently associated with each other.

Nature of low M_r components associated with CD55 and CD59 Ag. The ^{125}I-labeled material coprecipitated with both CD55 and CD59 Ag migrating on SDS-PAGE with the dye front could be detected if the gel was only briefly fixed/stained. It was lost upon prolonged washing of the gel in the destaining solution (45% methanol, 10% acetic acid). It could be selectively extracted from the immunoprecipitates by chloroform/methanol (2:1) (Fig.

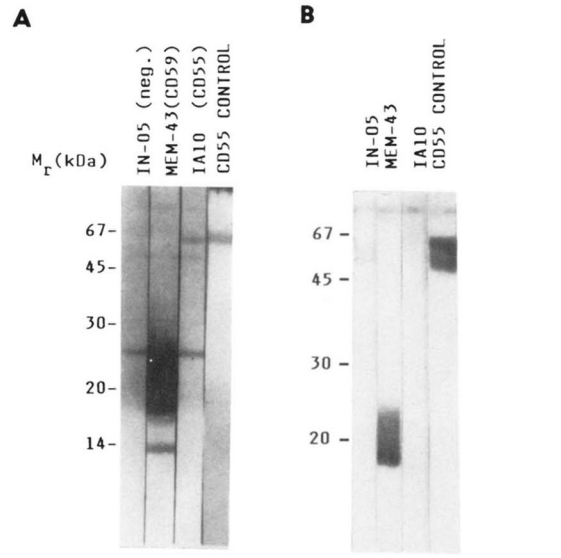

Figure 4. SDS-PAGE (15% gel) and Western blotting of the material isolated by immunoaffinity chromatography on immobilized mAb MEM-43 (CD59) from the HPB ALL (*A*) and HeLa cells (*B*). Individual strips were immunoperoxidase stained by an irrelevant negative control (IN-05), MEM-43 and IA10 (CD55) mAbs. The strip "CD55 control" in the part (*A*) is the Western blot of the CD55 Ag isolated from the HPB ALL lysate by solid phase immunoisolation and immunoperoxidase stained by the IA10 mAb; analogous strip in part (*B*) is Western blot of the HeLa cell lysate stained by the IA10 mAb. It is not clear why the HeLa cell CD55 appears to be more heterogeneous.

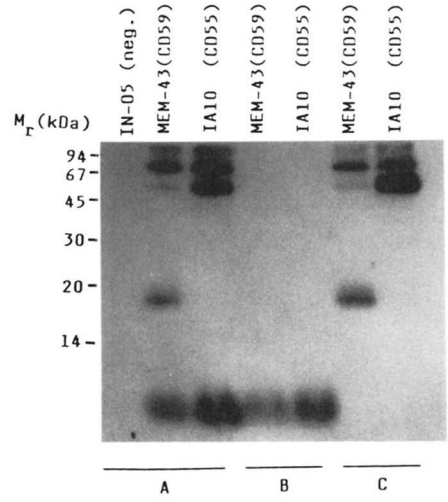

Figure 5. SDS-PAGE (15% gel) and autoradiography of the Ag CD59 and CD55 immunoprecipitated from detergent lysate of ^{125}I-labeled HPB ALL cells (*A*); the material extracted from these immunoprecipitates by chloroform/methanol as described in *Materials and Methods* (*B*); the material remaining after the extraction (*C*).

5). The chloroform-methanol extracted material could be separated into several components by TLC; the TLC patterns of the materials extracted from the CD55 and CD59 immunoprecipitates were very similar (Fig. 6). All these properties indicate that the low M_r material is probably a mixture of (glyco)lipids. The radioactive label is obviously incorporated into the lipid molecules by the familiar reaction of iodine with double bonds of unsaturated fatty acids.

Properties of 80-kDa component: comparison with CD44 glycoprotein. M_r of the 80-kDa component apparently associated with the CD55 and CD59 glycoproteins and its behavior on reduction and deglycosylation (shown above) were reminiscent of the CD44 glycoprotein (29) (I.

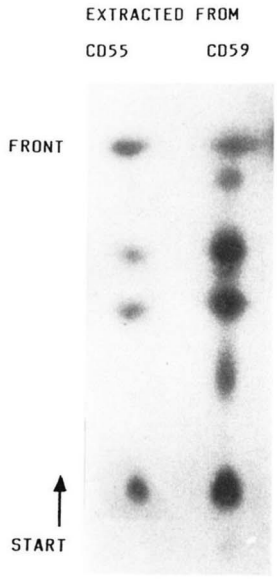

Figure 6. TLC and autoradiography of the material extracted from the CD59 and CD55 immunoprecipitates. Migration direction is indicated by the *arrow*.

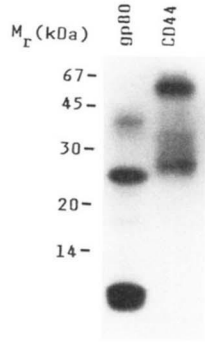

Figure 7. SDS-PAGE (15% gel) and autoradiography of the V8 protease digests of the gp80 (associated with CD59) and CD44 antigen immunoprecipitated from ^{125}I-labeled HPB ALL cells and RBC, respectively.

Štefanová, unpublished observations). However, several anti-CD44 mAb tested did not stain the HPB ALL cells in indirect immunofluorescence (not shown) and immunoprecipitated very little or no Ag from the detergent lysate (an example see in Fig. 1). The patterns of zones after V8 protease cleavage of the 80-kDa zone and of the CD44 Ag immunoprecipitated from E were quite different (Fig. 7) indicating that the 80-kDa component is not CD44.

Complexes associated with other GPI-linked gp. As shown in Figure 1, an anti-CD48 mAb immunoprecipitated a complex of composition similar to those observed in the case of CD55 and CD59. However, the 45-kDa CD48 Ag itself was poorly detectable presumably due to poor labeling. The pattern of the zones observed after SDS-PAGE of the material immunoprecipitated by an anti-Thy-1 mAb from the detergent lysate of radioiodinated murine thymoma EL-4 cell line was also strikingly similar to that yielded by previously shown mAb against human CD59, CD55, and CD48 Ag: in addition to the Thy-1 Ag (27 kDa), the 80-kDa major component as well as the (glyco)lipid zone were observed (not shown).

Protein kinase activity coprecipitated with GPI-linked gp. Addition of [γ-^{32}P]ATP to the immunoprecipitates obtained with various mAb resulted in specific phosphorylation of at least two major zones only in the cases of CD55, CD59, and CD48 but not in a number of other immunoprecipitates (Fig. 8). The apparent M_r of the 80-kDa zone in the immunoprecipitates of radioiodinated lysate vs in vitro phosphorylated immunoprecipitate is strikingly similar (Fig. 8*B*).

DISCUSSION

Our results demonstrate that at least two functionally important GPI-linked human leucocyte surface gp, CD55 and CD59, are associated on the HPB ALL cell surface with each other, with an as yet unidentified 80-kDa gp and with certain (glyco)lipids. This kind of complexes can be probably formed more generally by other GPI-linked surface proteins, as suggested by our results with CD48 and Thy-1 Ag. Several aspects of this phenomenon should be pointed out.

1) So far nothing is known about actual size and exact composition of these complexes on the cell surface and after detergent solubilization. They could be specific membrane areas, enriched in the GPI-linked glycoproteins, the 80-kDa component and relatively tightly bound lipids. Other components, e.g., of intracellular origin may be also present in such complexes, that were not detected in our experiments simply because they were not sufficiently labeled. We have noted that different anti-CD59 (directed against different epitopes) markedly quantitatively differed in their ábility to coprecipitate the additional components with the respective antigens (data not shown) although they precipitated essentially identical amounts of the 18-kDa CD59 gp. This may either indicate that some epitopes are poorly accessible in the complexes (and that substantial fraction of CD59 may exist in a free, uncomplexed form), or that binding of some mAb may partially disrupt the complexes.

2) The major 80-kDa component present in the complexes is a gp containing a 5 to 10 kDa N-glycosidically bound carbohydrate moiety of the complex type resembling closely the CD44 Ag. However, the results of V8 protease mapping clearly show that it is different from CD44. Other leucocyte molecules of similar M_r are CD18,

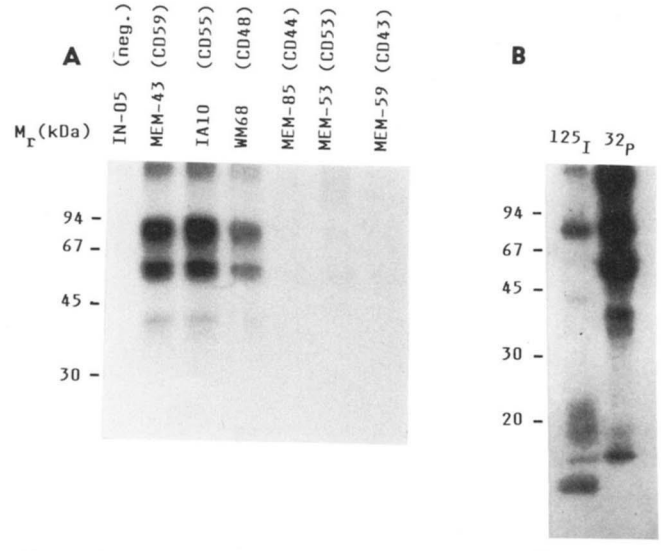

Figure 8. SDS-PAGE (15% gel) and autoradiography of the indicated immunoprecipitates after their incubation with [γ-^{32}P]-ATP (*A*). Negative results such as those shown in the last three lanes were obtained also in the cases of mAbs against b2m, CD2, CD3, CD4, CD7, CD8, CD29, CD18, CD45, and CD71 (not shown). Comparison of electrophoretic mobilities of the components immunoprecipitated from radioiodinated lysate and radiophosphorylated immunoprecipitate by the MEM-43 (CD59) mAb (*B*).

CD19, CD36, CD39, CD43 or CD54, for example. These also cannot be identical to gp80 either because they are absent from HPB ALL (CD19, CD36, CD39, CD54) or their M_r and behavior on deglycosylation are clearly distinct (CD18, CD43) (see Fig. 1). In murine cell lines, retroviral gp70 is a ubiquitous membrane component that is often present in immunoprecipitates and can be apparently associated with Thy-1 (30). However, in human cell lines similar gp of viral origin have not been observed (31). Therefore, we suggest that gp80 is a novel so far undescribed component of the leucocyte surface. At this moment we do not know whether it is also GPI anchored. Its closer characterization would be greatly aided by development of gp80-specific mAb.

3) These complexes seem to exist or to be stable enough only in some cell types. It remains to be determined whether their existence is restricted, for example, to normal counterparts of the HPB ALL and EL-4 cells, i.e., thymocytes, or to certain types of leukemic and lymphoma cells.

4) Functional relevance of these complexes is at present merely a matter of speculation. It has been difficult to understand how signals can be transmitted through the GPI-anchored molecules. It is actually striking that mAb of suitable epitope specificity against many GPI-anchored Ag exhibit activating effects (14, 32, 33). Association with a common molecule(s) such as the gp80 described here could be a clue to this puzzle.

5) Lipids are of course naturally associated with all membrane proteins but this association seems to be usually loose and it is lost upon standard solubilization in detergent solutions. The reason why the lipids are apparently more strongly associated with the present type of complexes is not clear. Also it is not known at present whether these are some specific lipids selectively accumulated in these complexes or whether their composition reflects the average composition of the membrane. Relatively tightly bound functionally relevant lipids were described in the case of high affinity IgE receptor (34), for example. Our observation that protein-associated lipids can be easily detected by SDS-PAGE of radioiodinated immunoprecipitates using a simple modification of the staining procedure may be useful in this kind of study. Our results also indicate that only a fraction of a protein Ag may be strongly associated with lipid molecules and that this fraction may be specifically recognized by some but not all mAb against that Ag. Although radioiodination is usually considered only as a method for protein (tyrosine) labeling, iodine is known to react avidly with double bonds of unsaturated fatty acids and therefore can also effectively label many membrane lipids. Exact nature of the low M_r components, presumably (glyco)lipids is yet to be determined; it is quite possible that this zone may also contain small hydrophobic peptides or lipoproteins. It should be noted that we observed similar low M_r zones in immunoprecipitates of well established glycolipid Ag such as CD15 and CDw17 (I. Štefanová, unpublished observations).

6) Of great potential interest is our observation of a kinase activity associated with the GPI-Ag-gp80-lipid complexes. The identity of the component of the complex exhibiting the enzymic activity is as yet unknown; possibly the 80-kDa component might be a self-phosphorylating kinase. Closer characterization of the kinase and demonstration of its substrate specificity remains to be done. Our preliminary results indicate that all components in the immunoprecipitates are phosphorylated exclusively on tyrosine residues (I. Štefanová, unpublished observations).

Several previously described observations may be relevant to our present results. First, murine Thy-1 gp was found to be functionally linked to an 85-kDa transmembrane gp and a 41-kDa G-protein further associated with cytoskeleton (35). Second, formation of exocytic vesicles sometimes called "exosomes" was described in tumor cells (36), reticulocytes (37), and E (38). These vesicles were in the latter case shown to be enriched selectively in two GPI-linked gp, CD55 (decay-accelerating factor) and acetylcholinesterase (39). This vesiculation appears to be a major route of Ag shedding (40). It could be speculated that our "complexes" are in fact precursors of such exosomes. Third, association of Thy-1 with CD45 and possibly other cell surface components has been recently observed (41); it will be interesting to evaluate the presence of CD45 in the immunoprecipitates obtained under the conditions used in this study. Finally, Thy-1 was reported to be associated with large detergent-resistant structures sedimenting during ultracentrifugation (42).

All these previous observations and our present data strongly indicate that GPI-linked membrane proteins are associated with other components; full elucidation of the nature, functional importance, and mutual relationship of these interactions remains yet to be done.

We are currently examining several issues raised by our study. It is necessary to define the cell types expressing the GPI-gp-gp80-lipid complexes, to examine in detail the nature and composition of these complexes and their possible relationship to the exosomes and to examine also other GPI-linked membrane proteins (e.g., CD14, CD16, CD24, CD58, CD67, and CD73) with respect to possible formation of similar complexes. An important question is to learn more about the gp80; to that aim we are currently trying to raise mAb against this molecule. It is also necessary to define the kinase activity associated with the complexes. We believe that clarification of these points will ultimately help to understand the functional relevance of these complexes.

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