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Isolation of plasma membrane domains from murine T lymphocytes

(subcellular fractionation/membrane protein/cell surface glycoprotein)

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ABSTRACT **Murine T-lymphoma cells have been homogenized in dense sucrose solution and centrifuged under isopycnic conditions for membrane components. Floating fractions banding between 10% and 22.5% sucrose ("light" membranes) and between 22.5% and 35% sucrose ("heavy" membranes) were shown to consist of smooth membrane vesicles. The amounts of cholesterol and phospholipids recovered after chloroform/methanol extraction were similar in both fractions, but heavy membranes contained two to three times more protein than light membranes. The most striking difference between the two membrane fractions was revealed by their labeled surface glycoprotein patterns on polyacrylamide gels, suggesting that (*i*) the smooth membrane vesicles originated from the plasma membrane and (*ii*) two distinct segments of the plasma membrane can be recovered in fractions characterized by specific surface glycoproteins. Light membranes were enriched in Thy-1 antigen, whereas Ly-5 antigen and a 170,000-dalton surface glycoprotein were recovered almost exclusively from heavy membranes, as were metabolically labeled protein spots comigrating with the H-2$^{k/d}$ antigen in two-dimensional electrophoresis. The patterns of the unlabeled proteins in light and heavy membranes appeared similar, except for polypeptides of 180,000 and 85,000 daltons that were found preferentially in heavy membranes. These results support the concept of plasma membrane domains by showing that two distinct populations of plasma membrane vesicles can be isolated and that these populations contain different sets of cell surface glycoproteins.**

In the fluid-mosaic model of membrane organization, a homogeneous lipid bilayer contains independently diffusible proteins (1). Departure from this uniform arrangement might occur when cytoskeletal elements transiently interact with select membrane proteins (2) or lipids (3) or both. Also, long-range interactions between proteins and lipids might induce specific lipid domains (4). Theoretically, both types of interactions could result in the delineation of membrane domains that have different biochemical and physical properties.

A limited number of studies have shown that plasma membrane fractions of different densities can be isolated from a single cell type (5–7). Although variations in the biochemical composition of these plasma membrane fractions have been reported (5, 6, 8), differences in their surface glycoprotein content have not been documented.

We have used two murine T-lymphoma lines bearing well-characterized surface markers (9) and isolated membrane fractions by sucrose density gradient centrifugation under isopycnic conditions for membrane components (7). Our results show that surface glycoproteins are by no means uniformly distributed among plasma membrane-containing fractions but rather segregate as unique groups into distinct membrane subfractions. The possibility that plasma membrane domains could be delineated by this procedure will be discussed.

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MATERIALS AND METHODS

Cells. BALB/c T-lymphoma cells Balentl 5 and P 1798 (Litton Bionetics) were passaged intraperitoneally in syngeneic mice. Lymphoma cells were recovered from ascitic fluids by washing in Hanks' medium and treated with 0.2 mM diisopropyl fluorophosphate (Fluka) before subcellular fractionation.

Labeling Procedures. For cell-surface labeling, 6×10^7 cells were incubated with 1 mCi (1 Ci = 3.7×10^{10} becquerels) of ^{125}I (New England Nuclear) as described (10); labeling at 4 or 20°C gave identical results. Biosynthetically labeled cells were recovered from an animal given 1 mCi of [^{35}S]methionine (prepared as described in ref. 11) intraperitoneally 15 hr before sacrifice.

Subcellular Fractionation. Two to 6×10^9 cells (1–3 ml of packed cells) were fractionated according to Monneron and d'Alayer (7) with the following modifications. One milliliter of packed cells was homogenized in (total vol, 6 ml) 60% sucrose in 50 mM Tris·HCl, pH 7.4/25 mM KCl/5 mM $MgCl_2$ (TKM medium). The homogenate was diluted to 40% sucrose with TKM medium, loaded as 9-ml aliquots in SW 27 tubes (Beckman), and overlaid with 9-ml volumes of 35%, 22.5%, and 10% sucrose solutions in TKM medium. Finally, 3 ml of TKM medium was layered on top of the 10% sucrose solution. Centrifugation under isopycnic conditions for membrane components (2 hr at 100,000 × *g*) was carried out as described (7). Floated membrane fractions (Fig. 1) were washed by dilution in TKM medium and sedimentation at 200,000 × *g* for 30 min.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis and Related Procedures. Membrane pellets were suspended in TKM medium and the proteins were precipitated with acetone and processed for one- and two-dimensional electrophoresis as described (10, 12). Silver nitrate staining of polyacrylamide gels was performed according to Switzer *et al.* (13). Actin and myosin markers were purified from P 1798 cells by following the procedure of Fechheimer and Cebra (14). For autoradiography, dried gels or nitrocellulose filters were exposed to Kodak SO-285 films in a Cronex (DuPont) cassette for 1–10 days and fluorography was carried out according to Bonner and Laskey (15). For the immunological identification of Thy-1 and Ly-5 antigens, membrane proteins were first electrophoretically transferred to nitrocellulose filters as described by Towbin *et al.* (16). The nitrocellulose blot of the two membrane fractions to be compared was incubated with a mixture of the heterologous antisera directed against the Thy-1 (17) and the Ly-5 antigens (10). The antisera were diluted 1:60 and 1:30, respectively. Bound antibody was revealed with ^{125}I-labeled protein A.

Electron Microscopy. Membrane fractions (see Fig. 1) were collected from gradients, fixed in suspension for 7 min at room temperature by adding equal amounts of 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and centrifuged at 30,000 rpm for 30 min. Pellets were washed in ice-cold cacodylate buffer

Abbreviation: kDal, kilodalton(s).

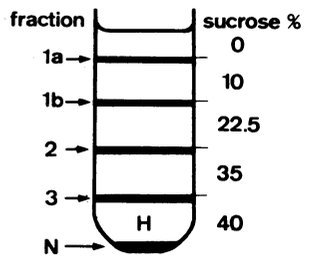

FIG. 1. Discontinuous sucrose gradient. H, homogenate; N, nuclei. Sucrose concentrations are % (wt/vol).

for 90 min, postfixed with collidine-buffered 1.3% OsO_4 for 30 min (4°C), stained in 2% uranyl maleate (pH 6) for 1 hr, dehydrated in a graded ethanol series, and embedded in Epon. Thin sections were stained with uranyl acetate/lead citrate and observed and photographed with a Philips 300 electron microscope.

Analytical Procedures. Protein determinations were performed according to Bradford (18). Lipids were extracted with chloroform/methanol according to Folch *et al.* (19); in this extract, cholesterol was measured as described by Moore *et al.* (20) and organic-phase phosphorus was determined as described by Bartlett (21). A multiplication factor of 25 was used to convert micrograms of organic phosphorus to micrograms of phospholipid.

RESULTS

Subcellular Fractionation and Electron Microscopy. The efficiency of cell breakage was equivalent to that reported for calf thymocytes (7), with more than 90% of the cells disrupted. The choice of sucrose concentrations for the discontinuous gradient was based on the banding characteristics of the floating membrane fractions on a 0–40% continuous sucrose gradient after overnight centrifugation. Three floating fractions were obtained with the discontinuous gradient (Fig. 1). Fraction 1a is contiguous to the lipid layer that accumulates at the meniscus. It contains lipid droplets and myelin figures (data not shown). This material can be effectively separated from the underlying membrane fraction by interposing a 10% sucrose layer so as to avoid contamination of fraction 1b with nonvesicular material. Fraction 1b bands between 10% and 22.5% sucrose (1.034–1.090 g/ml) and fraction 2 bands between 22.5% and 35% sucrose (1.090–1.136 g/ml). Electron microscopic examination shows that both fraction 1b and fraction 2 consist of closed membrane vesicles (Fig. 2) and that they display similar heterogeneity in size (diameter, 0.3–1.1 μm). The vesicles appear empty and devoid of attached filamentous or fuzzy material. Contaminants such as myelin figures, mitochondria, rough endoplasmic reticulum, and ribosomes are equally rare in both fractions. Mitochondria and, especially, rough microsomal vesicles can be recovered from fraction 3 and nuclei can be recovered from the gradient pellet (data not shown). Because fractionation of calf thymocytes by this method has shown that plasma membranes are enriched in bands corresponding to our fractions 1b and 2 (7), we focused our analysis on these two fractions, which will be referred to as "light" (fraction 1b) and "heavy" (fraction 2) membranes.

Biochemical Composition. The major difference between light and heavy membranes resides in their protein content (Table 1). Heavy membranes contain two to three times more protein than light membranes but comparable amounts of both cholesterol and phospholipids. This is reflected in the higher cholesterol/protein and phospholipid/protein (wt/wt) ratios of light membranes. The lipid compositions of light and heavy membranes, on the other hand, are similar, in that their cholesterol/phospholipid (mol/mol) ratios fall within the same range of values (0.81–1.05) for both lymphoma cell lines.

Characterization of Membrane Proteins and Glycoproteins. As shown in Fig. 3 (lanes a and b), the overall silver stained patterns of Balentl 5 light and heavy membranes are broadly similar and the major membrane polypeptides are present in both fractions. This also holds true for the P 1798 lymphoma line (data not shown). Among cytoskeletal proteins, actin and myosin (which were identified by comigration with purified markers) are also represented in both fractions. However, polypeptides of 180 and 85 kilodaltons (kDal) are markedly enriched in heavy (Fig. 3, lane b) and almost absent from light (lane a) membranes.

After vectorial labeling of surface-exposed glycoproteins, a much clearer distinction can be achieved between the two membrane fractions. By comparing autoradiograms of the patterns of light and heavy membrane proteins obtained by frac-

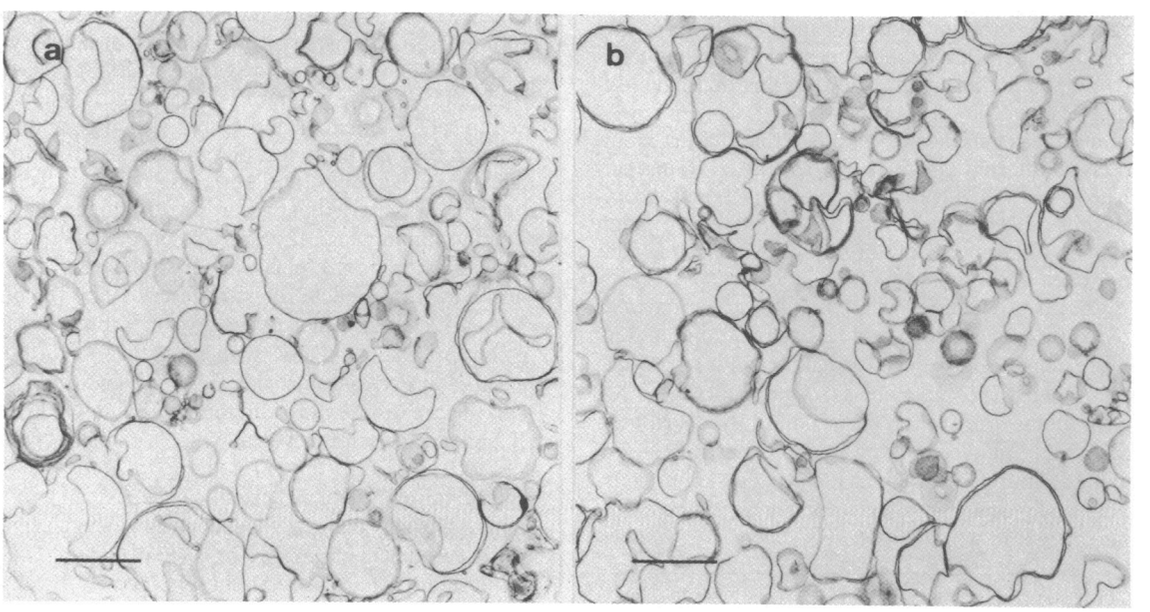

FIG. 2. Electron micrographs of light (*a*) and heavy (*b*) membrane fractions of P 1798 cells. Bars = 1 μm.

Table 1. Biochemical composition of membrane fractions

Cell line	Membrane	Protein, μg	Cholesterol, μg	Phospholipids, μg	Ratio: Cholesterol/ phospholipid (mol/mol)	Ratio: Cholesterol/ protein (mg/mg)	Ratio: Phospholipid/ protein (mg/mg)
Balentl 5	Light	85.9	38.8	95.4	0.81	0.45	1.11
	Heavy	153.9	40.0	75.7	1.05	0.26	0.49
P 1798	Light	74.8	44.2	89.3	0.98	0.59	1.19
	Heavy	181.9	45.3	88.6	1.01	0.25	0.48

Results correspond to the material recovered from 2 ml of packed cells. Values given are means of at least three separate experiments and the SEM is 15% of each value.

tionation of surface-labeled Balentl 5 and P 1798 cells (Fig. 3, lanes c–f), we found that bands of 170 and 180 kDal (comigrating with the silver-stained 180-kDal band) are recovered almost exclusively in heavy membranes (lanes d and f). A 90-kDal labeled band is found preferentially in heavy membranes of P 1798 (lane f). In the lower mass range, light membranes are enriched in a 25- to 30-kDal labeled band (lanes c and e). Quantitative estimates of the amounts of ^{125}I label in the 170- to 180-kDal and 25- to 30-kDal regions recovered from polyacrylamide gels of light and heavy membranes obtained from both cell lines are given in Table 2.

Straightforward comparison of the patterns of light and heavy membrane proteins in the two cell lines is no longer possible in the 40- to 65-kDal region, where Balentl 5 and P 1798 cells display altogether different patterns. Light membranes of Balentl 5 cells are enriched in a 50-kDal band (Fig. 3, lane c). Heavy membranes of P 1798 cells exhibit an intense, but diffuse, labeling between 40 and 65 kDal (lane f) that is fainter in the corresponding light membrane pattern (lane e).

Because the major surface-labeled components distinguishing light from heavy membranes correspond in electrophoretic mobility to the Ly-5 (180-kDal) (22) and Thy-1 (25- to 30-kDal) (23) antigens, we used antisera directed against Ly-5 (10) and Thy-1 (17) to identify these antigenic determinants on electrophoretic blots of total proteins from light and heavy membranes. The light membrane blot (Fig. 3, lane g) shows much more intense ^{125}I-labeled protein A labeling in the 25- to 30-kDal region than the heavy membrane blot (lane h), which, instead, displays protein A labeling at 180 kDal. This distribution is similar to the distribution of surface label at 180 and 25–30 kDal in light and heavy membranes, indicating that the Ly-5 antigen is carried by the 180-kDal band and the Thy-1 antigen, by the 25- to 30-kDal band.

Given the differences in surface labeling observed between light and heavy membranes in the 40- to 65-kDal range (Fig. 3, lanes c–f), it was of interest to see whether the H-$2^{k/d}$ antigen (48–54 kDal) accumulated in one or the other type of membrane. Therefore, we compared two-dimensional maps of both membranes for the presence of the H-2 antigen after metabolic labeling with [^{35}S]methionine, which has been shown to label it efficiently (24). The results shown in Fig. 4 were obtained with Balentl 5 cells and identical maps were obtained with P 1798 cells (data not shown). A series of six spots (Fig. 4*b*, asterisks) with increasing molecular masses and decreasing isoelectric points is clearly visible in the heavy-membrane map but absent from the light-membrane map (Fig. 4*a*). Additional spots are selectively present in the heavy-membrane map (Fig. 4*b*, arrows) that have isoelectric points similar to the most acidic components of the six-spot series. Strongly labeled spots (Fig. 4*b*, arrowheads) within the basic end of the group are also more abundant in heavy membranes. All other spots on both light- and heavy-membrane maps constitute a common pattern (Fig. 4), except for the spots contained within a cluster between 30 and 35 kDal (Fig. 4*a* and *b*, brackets). The fact that the six spots designated by the asterisks closely resemble the two-dimensional pattern of H-2^k and the three spots marked by arrows

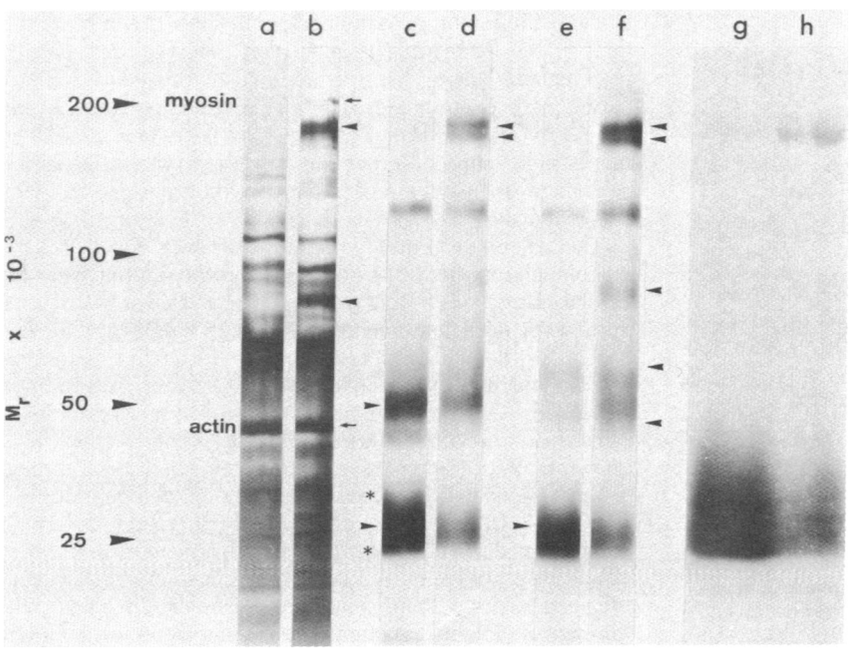

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis (5–20% acrylamide gradient gel) of light and heavy membrane proteins. Lanes: a and b, silver staining patterns of 20 μg of Balentl 5 membrane proteins from light and heavy membranes, respectively; c–f, autoradiograms of membrane proteins from ^{125}I-surface-labeled cells (c, Balentl 5, light membranes, 20 μg, 30,000 cpm; d, Balentl 5, heavy membranes, 20 μg, 30,000 cpm; e, P 1798, light membranes, 25 μg, 27,000 cpm; f, P 1798, heavy membranes, 25 μg, 27,000 cpm); g and h, autoradiogram of a nitrocellulose blot containing membrane proteins from P 1798 cells after incubation with a mixture of heterologous anti-Thy-1 (1:60) and anti-Ly-5 (1:30) antisera, followed by ^{125}I-labeled protein A (g, light membranes, 70 μg; h, heavy membranes, 70 μg). Arrowheads indicate positions of polypeptides enriched in light or heavy membranes. Arrows indicate positions of actin and myosin. Asterisks define the portion cut out for determination of radioactivity of the 25- to 30-kDal band (Table 2).

Table 2. Distribution of surface-labeled components among membrane fractions

Cell line	Membrane	Radioactivity, cpm	
		25–30 kDal	170–180 kDal
Balentl 5	Light	2,637	177
	Heavy	1,392	522
P 1798	Light	2,070	269
	Heavy	1,107	1,538

^{125}I radioactivity was excised from the dried gel of Fig. 3 (lanes a and b) on the basis of the autoradiograms of this same gel shown in lanes c and d (Balentl 5 cells) and lanes e and f (P 1798 cells). An area corresponding to the radioactivity detectable above and below 25 kDal (between the two asterisks of lane c) was excised from lanes c–f (Fig. 3). For the 170- and 180-kDal bands, radioactivity corresponding to the arrowheads on lanes d and f was excised from lanes c–f.

resemble the pattern of the most acidic H-2^d components (24) suggests that the majority of the H-2$^{k/d}$ glycoproteins segregate with heavy membranes rather than with light membranes.

DISCUSSION

Two floating fractions enriched in smooth membrane vesicles have been obtained from T-lymphoma cells after homogenization in dense sucrose solution followed by isopycnic centrifugation of membrane components. As previously shown by fractionation of calf thymocytes by an analogous method, contamination of floating membrane vesicles with intracellular smooth membranes always occurs (7). However, the enrichment in ectoenzymes showed that calf thymocyte membrane fractions corresponding to our light and heavy membranes consisted essentially of plasma membranes (7). Moreover, the surface antigens followed in this study represent unambiguous cell-surface markers because they are sialylated glycoproteins (10, 12). Also, the two-dimensional patterns of metabolically labeled spots

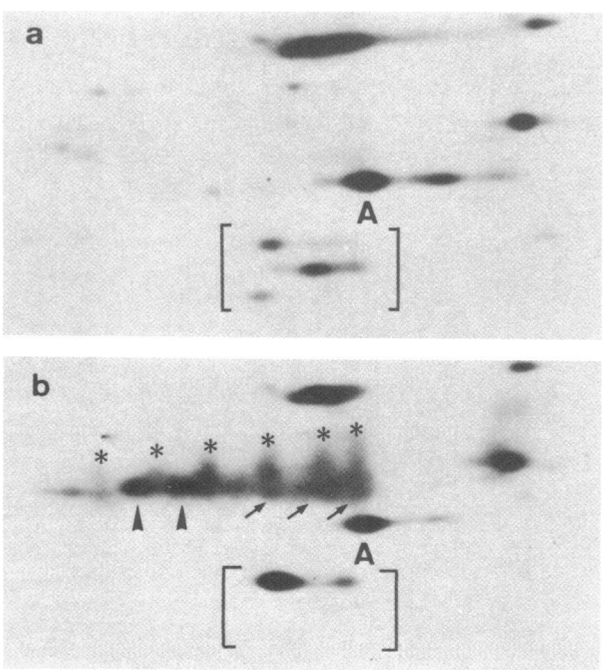

FIG. 4. Two-dimensional electrophoresis of *in vivo* [^{35}S]methionine-labeled membrane proteins from Balentl 5 cells. (*a*) Light membranes. (*b*) Heavy membranes. The acidic side is to the right. Asterisks indicate the H-2^k spots. Arrows indicate the H-2^d spots. Arrowheads and bracket, see text. A, β-actin.

comigrating with the H-2 antigen that we recover from heavy membranes are clearly distinguishable from pulse-labeled H-2 precursors and represent a typical pattern of mature sialylated H-2 glycoproteins (24).

The differences in density between membrane fractions are most likely explained by variations in the lipid/protein ratios; the heavy membrane fraction contains two to three times more protein than the light membrane fraction. The cholesterol/phospholipid ratio, on the other hand, is similar in light and heavy membranes. Therefore, it appears that two compartments of plasma membrane separate into distinct fractions on centrifugation, based on the amount of protein they contain. The question then arises as to whether light and heavy membranes could consist of distinct proteins having different relationships with their membranous or cytoplasmic environments. Judging from electron microscopic examination, preferential association of one type of membrane with cytoplasmic filaments appears unlikely, since membrane vesicles from both fractions are free of attached material (Fig. 2). By gel analysis, the silver-stained protein patterns of light and heavy membranes resemble each other throughout the molecular mass range examined, and actin and myosin can be detected in similar amounts in both fractions. Detectable exceptions are the polypeptides of 180 and 85 kDal that are present in the heavy, but absent from the light-membrane pattern (Fig. 3, lanes a and b) and the biosynthetically labeled proteins of 30–35 kDal that form distinct patterns in each fraction (Fig. 4).

The most striking difference between light and heavy membranes is illustrated by their autoradiographic gel patterns after cell-surface labeling (Fig. 3); in both cell types, Thy-1 accumulates in light membranes whereas Ly-5 and a 170-kDal surface-labeled component are preferentially recovered with heavy membranes, together with components similar to H-2$^{k/d}$ by two-dimensional electrophoresis. Interestingly, the two lymphoma lines compared in this study [which have been reported to differ in their surface antigen compositions (9)] exhibit differences between the surface-labeled glycoprotein patterns of their light membranes (especially in the 50- to 65-kDal range) whereas their heavy membranes appear to be similar.

The membrane fragments generated during homogenization tend to reseal and possibly reassociate into vesicles of different sizes. Because the two membrane fractions recovered from the gradient still contain distinct sets of surface glycoproteins, it is likely that different membrane fragments have been produced that, to the extent that reassociation occurred, reassociated preferentially among like fragments. This procedure for preparing plasma membranes therefore reveals structural heterogeneity in this organelle by yielding two classes of resealed fragments containing different surface glycoproteins and thus releasing what may be regarded as two different plasma membrane domains.

The major glycoprotein in light membranes, Thy-1, is a peripheral glycoprotein that does not span the lipid bilayer (25). Structural analysis of this polypeptide has shown that it lacks an obvious intramembranous hydrophobic segment (25). It is thought to be anchored in the bilayer through a nonprotein hydrophobic tail that links its COOH terminus to membrane lipids (25). On the other hand, the surface glycoproteins in which heavy membranes are enriched (Ly-5 and H-2) have been shown to be transmembrane glycoproteins, anchored through a well-defined hydrophobic segment of their polypeptide chain (26, 27). Such differences in membrane insertion could be of importance during homogenization in that membrane breakage would be more likely to occur where oligomers of transmembrane proteins delimit membrane channels (28) rather than through membrane segments containing nontransmembrane

Thy-1 glycoproteins. These membrane segments, by virtue of the known affinity for lipids of Thy-1 (25), could conceivably represent distinct lipid domains (4) and therefore segregate from other membrane fragments containing transmembrane proteins.

Explanations for this segregation implying patching or capping of surface glycoproteins prior to homogenization can be ruled out because no redistribution occurs in the cold at the surface of the lymphoma cells studied unless external ligands to surface glycoproteins are added.

Thy-1 also differs in another respect from both H-2 and Ly-5 in its relationship to the plasma membrane. We have reported that part of the Thy-1 molecules remain in a sedimentable form after nonionic detergent treatment of whole cells (29). Preliminary evidence suggests that such insoluble Thy-1 could interact with other insoluble components of the plasma membrane and therefore be part of the plasma membrane matrix (cf. ref. 30). Whether interactions of this type between Thy-1 and the plasma membrane matrix could influence the compartmentalization and vesiculation of the lymphocyte plasma membrane will require further study.

In summary, we have evidence that at least two different domains constitute the T-lymphoma cell membrane. These domains consist of a qualitatively similar, but quantitatively different, set of membrane proteins in lipid bilayers of similar cholesterol and phospholipid content. Surface glycoproteins represent the distinctive elements between domains, as Thy-1 accumulates in the protein-depleted light membranes whereas Ly-5 (180 kDal), a glycoprotein of 170 kDal, and H-$2^{k/d}$ segregate in the protein-rich heavy membranes.

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