Dynamics and Longevity of the Glycolipid-anchored Membrane Protein, Thy-1

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Abstract. Thy-1 and a number of other proteins are anchored to the outer hemi-leaflet of membranes by a glycolipid moiety containing ethanolamine phosphate, mannose, glucosamine, and phosphatidylinositol. They nevertheless have the striking property of being able to transduce signals across the plasma membrane. We here demonstrate, for the BW5147 murine T lymphoma, that (*a*) >90% of Thy-1 is at the cell surface, (*b*) Thy-1 is about one order of magnitude less concentrated in coated pits than the transferrin receptor or H-2 antigens, (*c*) Thy-1 undergoes at most very limited endocytosis or diacytosis, and (*d*) Thy-1 has an unusually slow turnover rate. Several similar observations have also been made for a second glycolipid-anchored protein, the T cell activating protein. Thus, the absence of cytoplasmic and trans-membrane domains may result in lipid-anchored proteins being confined to the cell surface and being free from constraints which affect the turnover of transmembrane proteins.

PROTEINS that are integrated into membranes by a glycolipid moiety, are found in protozoa, yeasts, and higher eukaryotes (9, 16, 32). Many of these proteins are sensitive to cleavage by microbial phosphatidylinositol-specific phospholipase C (PI-PLC).[1] It has therefore been suggested that such anchors may be cleaved by the cells that bear them on their surface or by neighboring cells and secretion products, thereby releasing potent intracellular messengers and extracellular polypeptides and modifying the cell surface (8, 22, 32, 43). An additional feature of lipid-anchored proteins, which offers a possible rationale for their existence, is the observation that certain of these proteins diffuse more rapidly in the plane of the membrane than proteins that bear transmembrane and cytoplasmic domains (16, 32). The generality of this conclusion has been questioned since certain lipid-anchored proteins are not more mobile than transmembrane proteins (6, 43).

The Thy-1 antigen of neurons, murine T lymphocytes, epithelial cells, and fibroblasts is one of the best studied glycolipid-anchored proteins (7, 28, 29, 37, 47, 53). The structures of its polypeptide and glycans (7, 41) and the anchor moiety (20) are known. Moreover, the binding of antibodies to appropriate epitopes on glycolipid-anchored proteins such as Thy-1 can lead to calcium influx and cell differentiation (13, 25, 26, 33, 44, 50). For Thy-1 to mediate such signals on T cells, the T cell receptor must be present (17, 52). Thy-1 has been reported to have a high mobility at the cell surface (14, 23) and to be poorly represented in coated pits (4). Using BW5147 murine lymphoma cells (18), we explore here the membrane dynamics of both Thy-1 and a second lipid-anchored antigen, [the T cell–activating protein, TAP], which is a member of the Ly-6 family of antigens and can also mediate signal transduction (1, 35, 64, 65).

1. *Abbreviations used in this paper*: PI-PLC, phosphatidylinositol-specific phospholipase C; TAP, T cell–activating protein; TfR, transferrin receptor.

Materials and Methods

BW5147.G.1.4 murine lymphoma cells (American Type Tissue Culture Collection, Rockville, MD), and the class C mutant (TIM1.7BU.2), and class E mutant BW5147.3/Thy-1.1$^-$.10G.OUA^R.1 (from R. Hyman, Salk Institute) were cultured in DME with 10% decomplemented horse serum and antibiotics in a 5% CO_2, 95% air humidified incubator.

All immunostaining was performed with samples of 2×10^6 washed lymphoma cells incubated in 0.1 ml of PBS-10 mM sodium azide or DME-10 mM Hepes supplemented in each case with 50 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Incubations with primary or secondary reagents were for 30 min on ice. Each incubation was followed by dilution with 1 ml albumin–PBS–azide, sedimentation, resuspension, and resedimentation.

The monoclonal antibodies used were in the form of diluted hybridoma culture supernatants which had been titrated to give a maximal signal on BW5147. For Thy-1 we used M5/49, a rat IgG2a (11), for TAP we used 3E7.1, a mouse IgG2b (gift of K. Rock, Harvard Medical School), for H-2K^k we used 11.4.1, a mouse IgG2a (40), and for the murine transferrin receptor we used R17.217.1.4, a rat IgG2a whose binding is not competed by transferrin (30). Secondary reagents were FITC-goat anti-mouse or anti-rat Ig at a dilution of 1:10 (Fisher Scientific Co., Pittsburgh, PA). Flow cytometry was performed with a Cytofluorograph 2S (Becton Dickinson & Co., Mountainview, CA). Dead cells were gated out with propidium iodide.

We have recently presented the flow cytometric procedure for quantitating surface and total cell-associated antigens in detail (60). Controls including nonspecific first antibody were included for each condition and their mean fluorescence was subtracted from that of the experimental samples.

Electron Microscopic Immunocytochemistry

Cells were washed in PBS, fixed with periodate–lysine–paraformaldehyde

pH 6.2, for 30 min on ice, washed in PBS, allowed to attach to poly-L-lysine-coated Petri dishes and permeabilized with freshly prepared 0.01% saponin (Sigma Chemical Co.) in PBS supplemented with 1 mg/ml BSA (5). They were then incubated for 4 h at 4°C with anti-Thy-1 (M5/49) in BSA-saponin-PBS, washed four times, incubated 4 h at 4°C with 1:200 horseradish peroxidase-conjugated Fab goat anti-rat Ig (Biosys), washed four times, refixed 1 h at 24°C in 1.5% glutaraldehyde, 5% sucrose, 0.1 M Na cacodylate-HCl pH 7.4, washed, incubated 30 min at room temperature with peroxidase substrates (2 mg/ml diaminobenzidine, 0.012% H_2O_2) supplemented with 1 mg/ml imidazole in 50 mM Tris-HCl pH 7.4, 7.5% sucrose, washed, refixed in fresh 9:1 1%OsO_4/1% $K_4Fe(CN)_6 \cdot 3H_2O$ for 1 h on ice, dehydrated, and embedded. Thin sections were examined without further staining.

Studies of the Distribution of Antigens at the Cell Surface

Cells were washed in DME and fixed at room temperature in 2% formaldehyde-0.1% glutaraldehyde in PBS for 20 min, washed with PBS, quenched with 50 mM NH_4Cl-PBS, incubated with monoclonal antibody (or none) for 30 min at room temperature, washed twice with PBS-0.5% bovine serum albumin-10 mg/ml sodium azide, and reincubated 2 h in PBS-albumin-azide supplemented with colloidal gold (10 or 15 nm diameter) conjugated to goat anti-mouse Ig or goat anti-rat Ig (Jansen Lab., Olen, Belgium), as appropriate. The cells were then washed twice, refixed with glutaraldehyde and osmium, dehydrated, and embedded in Spurr. Thin sections stained with lead citrate and uranyl acetate were examined in the electron microscope. For quantitative evaluation of antigen distribution, individual cells were scanned and photographed systematically at 10,000× to tabulate the total number of gold particles bound to the cell perimeter, the number of coated pits per perimeter, and the number of gold particles within coated pits per cell. In the absence of specific monoclonal antibody, no surface label was seen.

Studies of Endocytosis

Cells were washed in PBS and resuspended at 1-10 × 10^6 cells/ml in ice-cold PBS pH 8.0 containing freshly dissolved sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL), 1 mg/ml (31). After 30 minutes at 4°C, 1 vol DME was added and the cells were then washed twice in PBS. Probing to quantitate surface biotin was achieved by incubating aliquots of 10^6 cells in 100 μl PBS-BSA (1 mg/ml) supplemented with 100-200,000 cpm ^{125}I-streptavidin (Amersham Corp., Arlington Heights, IL), brought to 1 μg streptavidin/ml with unlabeled streptavidin (Boehringer Mannheim Diagnostics, Houston, TX). After 30 min at 4°C with shaking, the cells were layered over 300 μl 20% sucrose in 40 mM Tris-HCl pH 7.4 and centrifuged 5 min at 4°C at top speed in an Eppendorf microfuge (Brinkmann Instruments Corp., Westbury, NY). The tubes were then snap frozen and cut to count both supernatant and pellet. Under these conditions, the amount of ^{125}I-streptavidin bound was a linear function of the number of biotinylated cells. About 20% of the ^{125}I was bound in typical experiments. In controls with cells that had not been biotinylated, ~1% of the cpm were recovered in the pellet.

Determination of Endocytosis of Lipid-anchored Proteins

Biotinylated cells were recultured for 5-120 min at 37°C and treated with PI-PLC of *Bacillus thuringiensis* at 0°C, as below. The resulting supernatants were ultracentrifuged and then both the clarified supernatant and cell pellets (dissolved in sample buffer after nuclei had been removed) were reduced and fractionated by SDS-PAGE. After blotting to nitrocellulose and blocking with nonfat dried milk and BSA, the blots were probed with ^{125}I-streptavidin in 0.5% Tween-20, 1 M glucose, 3 mg/ml BSA, 10% glycerol in PBS. The blots were autoradiographed and subsequently cut and counted in a gamma counter. Thy-1 and an unidentified protein with an apparent molecular mass of ~130 kD were consistently detected in the PI-PLC-released material. To control for the possible effects of biotinylation on endocytosis, control and biotinylated cells were incubated 30 min at 0°C with rhodamine-human transferrin (gift of F. Maxfield, Columbia University), washed, and reincubated 5 min at 37°C. In both cases, comparable major internalization of rhodamine was seen.

Studies of Diacytosis

PI-PLC, trypsin (TPCK-treated; Sigma Chemical Co.), and Pronase (Boehringer Mannheim Diagnostics) were used. Cells were treated with 0.1 ml of enzyme solution for 60 min at 0°C. The cells were then supplemented with 1 ml of DME containing 10% fetal bovine serum, 1 mg/ml DNase (Sigma Chemical Co.), and 2 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.), sedimented, washed in the presence of serum, and reincubated 1 h at 0 or 37°C. They were then washed in serum-free DME and stained for surface antigens. The enzyme doses selected for use at 0°C (the lowest dose giving maximal effect) were 10 μmol PI U/min per milliliter PI-PLC for Thy-1 and TAP (used in Serumless Medium [Gibco Laboratories, Grand Island, NY]-20 mM Hepes-5 × 10^{-5} M betamercaptoethanol), 1 mg Pronase/ml DME for TAP and 1 mg Trypsin/ml DME for TfR.

Studies of Thy-1 Turnover

0.5 × 10^6 cells were labeled 17 h in 0.5 ml DME-5% serum containing 0.5 mg/ml glucose and 100 μCi 1,6-[^{3}H]glucosamine (NET-557; 44.8 Ci/mol; Dupont-NEN Products, Wilmington, DE). The cells were washed twice in complete medium (DME-5% serum-antibiotics) and maintained in culture for 47 h at 0.25-1 × 10^6 cells/ml. A second sample of 0.5 × 10^6 cells was labeled 17 h with 10 μCi 1-[^{14}C]glucosamine (NEC-193; 10 mCi/nmol; Dupont-NEN Products). The two cell populations were then pooled, washed twice in complete medium, and recultured 3 h in DME-serum supplemented with 1 mM glucosamine (Sigma Chemical Co.) to allow full transport to the plasma membrane of newly synthesized Thy-1 (which was confirmed in pilot PI-PLC release experiments). At this point the cells were washed and treated with PI-PLC at 0°C as above. The resulting supernatant was clarified by ultracentrifugation and both the supernatant and an aliquot of the cell pellet were reduced and analyzed by SDS-PAGE along with molecular weight standards on a 17% gel which was fixed and stained. Both lanes were subsequently sliced into 0.25-cm slices, which were hydrolyzed in 0.5 ml 30% H_2O_2 at 47°C for 1 d and counted 10 min in 25% Triton X-114-0.8% butyl-PBD (Dupont-NEN Products)-75% xylene using an appropriate ^{3}H-^{14}C double-label program in a liquid scintillation counter (Beckman Instruments Inc., Palo Alto, CA).

Results

To evaluate fractional surface expression of Thy-1, TAP, H-2, and TfR, cells were fixed and stained for flow cytometry in the absence or presence of the detergent, saponin. Fig. 1 shows that 90% of cell-associated Thy-1 and TAP is accessi-

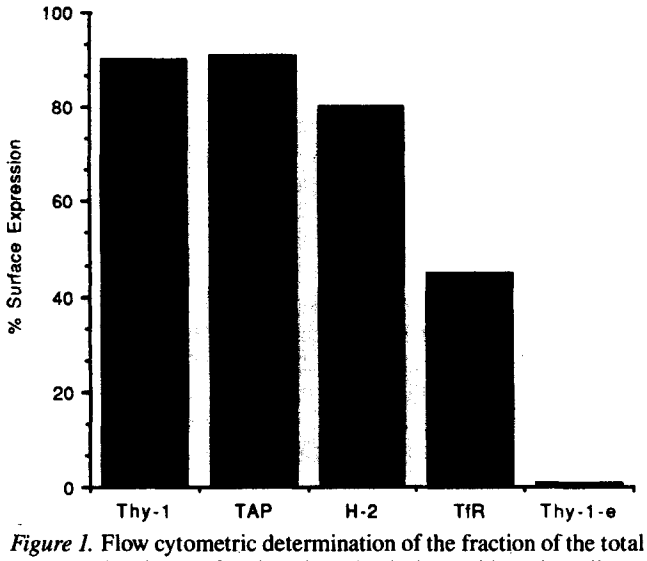

Figure 1. Flow cytometric determination of the fraction of the total cell-associated pool of each antigen that is detectable at the cell surface. Both lipid-anchored antigens are 90% patent, while H-2 and TfR are less accessible before permeabilization. The last column presents a corresponding result for Thy-1 expression by the class E lymphoma mutant. As expected (15), no surface Thy-1 is detectable. Vertical axis indicates the percent surface expression, i.e., specific signal in the absence of saponin ÷ signal with saponin.

ble in the absence of saponin, unlike H-2 and TfR, for which larger latent pools exist.

An independent qualitative assessment of Thy-1 localization was obtained by electron microscopic immunocytochemistry (Fig. 2). As shown, Thy-1 is detected only at the cell surface. The adequacy of permeabilization is verified by parallel examination of a mutant lymphoma, which does not add a lipid anchor or express surface Thy-1 (10, 15). In these cells Thy-1 is readily detected in the nuclear envelope but not at the cell surface.

To evaluate the distribution of lipid-anchored proteins in the plane of the plasma membrane, fixed cells were incubated with appropriate monoclonal reagents followed by colloidal gold conjugates. As indicated in Table I, Thy-1 and TAP are only infrequently found in coated pits. By contrast, TfR and H-2 are severalfold enriched in coated pits. Coated pits constitute 1–2% of the surface of lymphoblastoid cells and fibroblasts (4, 12, 51).

To evaluate the average kinetics of endocytosis of surface antigens, cells were surface biotinylated and either held on ice or reincubated at 37°C for 5–120 min. Each sample was then surface probed at 0°C with ^{125}I-streptavidin, or treated with PI-PLC at 0°C to release lipid-anchored proteins. In the latter case, each supernatant was resolved by SDS-PAGE, blotted onto nitrocellulose, and the blots were probed with ^{125}I-streptavidin. As shown in Fig. 3 *right*, the average signal diminished only extremely slowly. PI-PLC consistently released biotinylated Thy-1 and small amounts of a protein of ~130 kD. The amount of Thy-1 released by PI-PLC did not obviously diminish during the course of the experiment (Fig. 3 *left*). Quantitative evaluation of these data is consistent with this conclusion and indicates that 3.5–5.8% of the cell surface biotin is released as PI-PLC-sensitive Thy-1. Control experiments (see Materials and Methods) showed that biotinylation did not block transferrin endocytosis.

To extend these observations, a set of "antigen replacement" experiments was performed. As a preliminary for these studies, cells were treated with PI-PLC on ice to remove maximal amounts of Thy-1 and TAP under conditions in which membrane recycling could not occur. Parallel experiments on TAP also used Pronase. To remove TfR, trypsin was used, but neither protease removed H-2.

As illustrated in Fig. 4, when essentially all TfR and TAP or 75% of Thy-1 were removed from the cell surface, reincubation for 1 h at 37°C lead to extensive regeneration of only TfR. Thus, if intracellular pools of the lipid-anchored proteins do exist, they must either be relatively small (~10%) or fail to recycle during the time interval studied.

To evaluate the relative turnover rate of lipid-anchored surface Thy-1, a two isotope labeling protocol was used. Cells were labeled overnight with [^{3}H]glucosamine followed by a 2-d chase incubation. A second lot of cells was labeled overnight with [^{14}C]glucosamine. The two were then pooled, chase incubated for 3 h, and treated with PI-PLC at 0°C. The resulting cell pellet and supernatant were analyzed by SDS-PAGE.

As illustrated in Fig. 5, labeled Thy-1 is readily detected among the released proteins and has a ^{3}H/^{14}C ratio about five times higher than other gel slices. Although labeled glucosamine may be converted to other sugars, or be reused, these observations prove that the glycans of Thy-1 turn over strikingly more slowly than the glycans of other labeled glycoproteins. The lack of labeling of TAP is presumably a reflection of its lesser abundance (also documented in Fig. 3 *right*) and very slow turnover.

Discussion

For T lymphocytes, qualitative microscopic studies that predate the discovery of lipid anchoring have shown that Thy-1

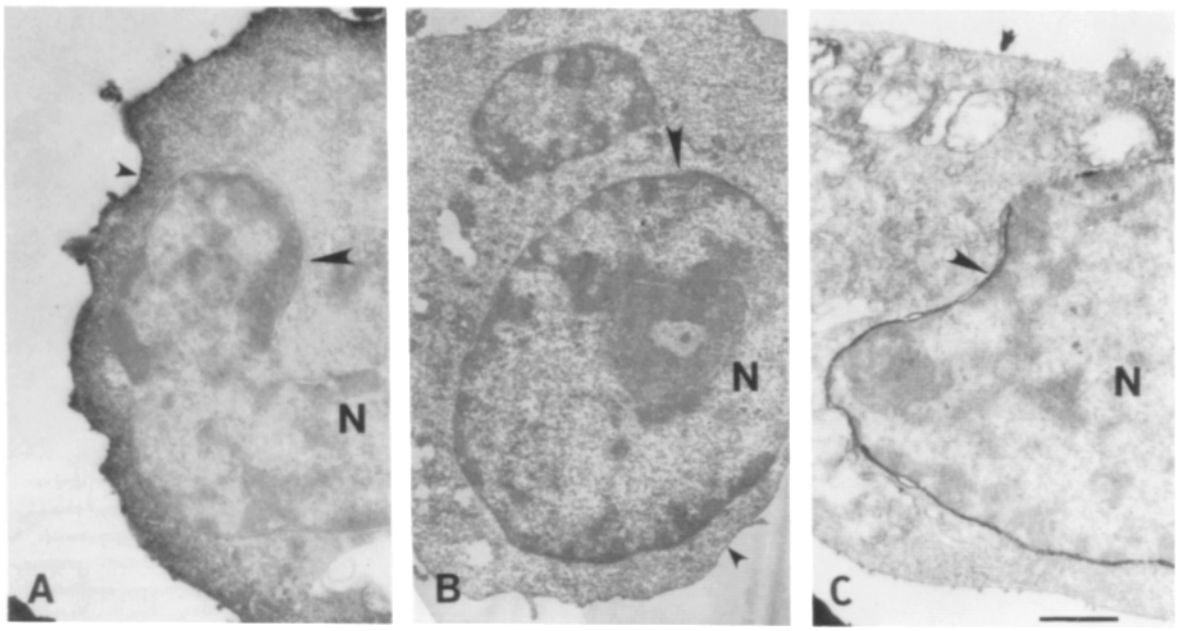

Figure 2. Electron microscopic immunocytochemical detection of Thy-1 in wild-type (*A* and *B*) and mutant (*C*) lymphomas. *B* is a control using a non-specific first antibody. On wild-type cells the peroxidase reaction product is at the cell surface. In the class C mutant it is restricted to the nuclear envelope. (*large arrowheads*). Small arrowheads, plasma membrane. Bar, 2 μm.

Table I. Antigen Representation in Coated Pits.

Antigen	No. of cells	No. of particles	Gold in pits
			%
Experiment 1			
Thy-1	30	1,991	0.7
TAP	61	1,586	0.4
H-2K^k	40	540	4.7
TfR	21	6,890	4.3
Experiment 2			
TfR	30	1,931	3.7

Lymphoma cells were fixed, incubated with monoclonal antibodies corresponding to the four antigens, and then incubated with appropriate colloidal gold conjugates. After postfixation and embedding, thin sections were examined for the presence of gold particles within the limits of coated pits. Both lipid-anchored antigens are underrepresented in pits, which account for 1–2% of the cell perimeter. With control antibody followed by gold conjugates essentially no surface label was seen (<40 particles on 20 cells counted).

and the lipid-anchored class I antigen, Qa, are concentrated at the cell surface (54, 58). By contrast, transferrin receptors, H-2, class II histocompatibility antigens, and T200 (34, 58) are also readily demonstrated at intracellular locations, which are presumably endosomes. Our quantitative studies show that at least 90% of two lipid-anchored proteins is at the plasma membrane.

Studies which predate the discovery of lipid anchoring of Thy-1 and 5′-nucleotidase have documented the poor representation of these (4, 36) and other antigens (39, 48) in coated pits, in contrast to H-2 and the TfR (19, 21, 34). In the present study we confirm these observations and show that a second lipid-anchored protein, TAP, is also underrepresented in coated pits. The presence of lipid-anchored proteins in coated pits may therefore be only transient, and result in their undergoing only infrequent "passive" internalization by contrast with many transmembrane proteins bearing suitable cytoplasmic domains (12, 27, 38, 45, 49, 62). Such internalization is postulated to be mediated by adaptors that interact both with clathrin and with the cytoplasmic domains of transmembrane proteins (42).

The observation that Thy-1 (and TAP) undergoes only limited endocytosis and diacytosis is striking, but does not distinguish it from transmembrane proteins, many of which are internalized only slowly on murine thymomas and other cells (46). It is known that lipid-anchored antigens can be

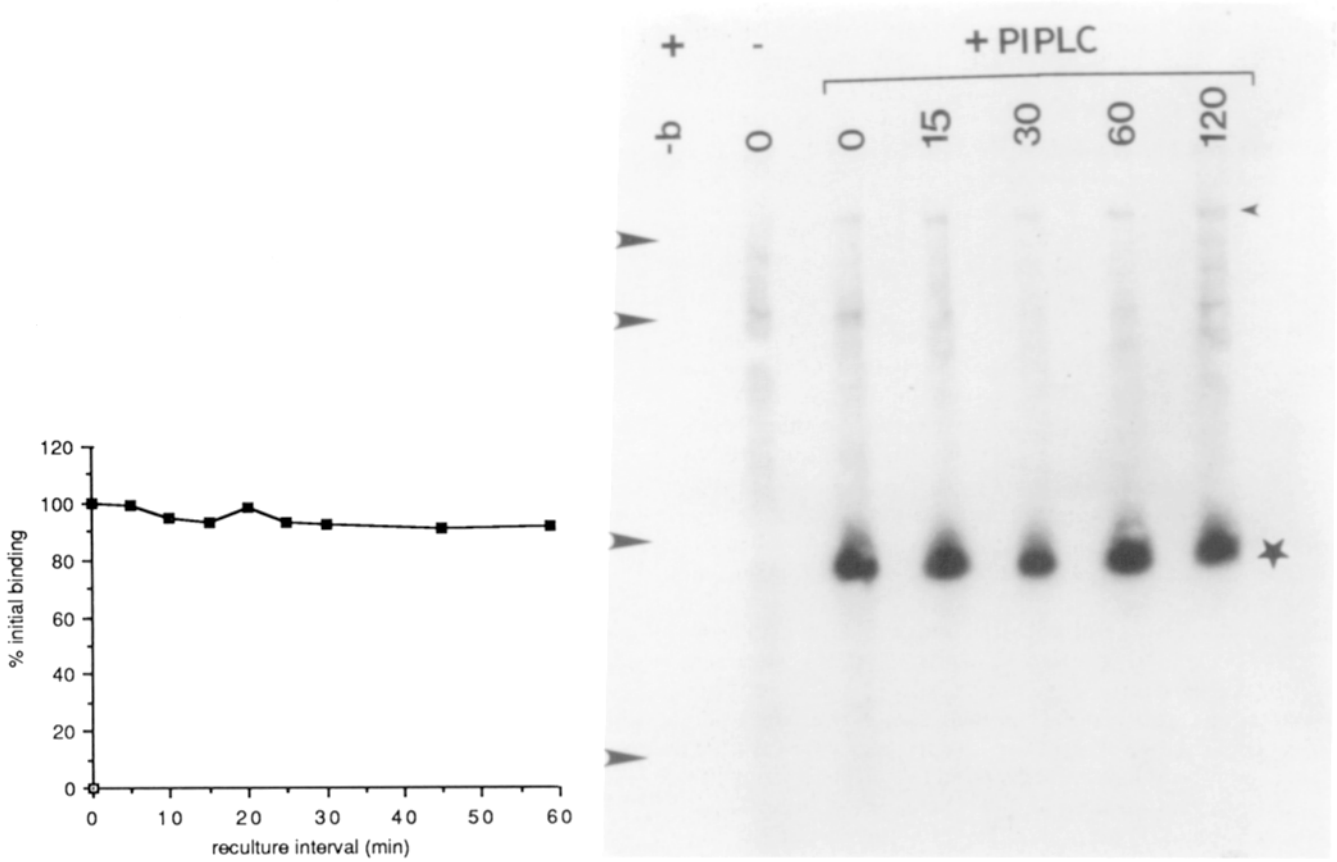

Figure 3. Evaluation of endocytosis of surface antigens. (*Left*) Cells were biotinylated at 0°C, washed, reincubated for increasing periods at 37°C, and then probed at 0°C with ^{125}I-streptavidin. As shown, the total cell surface signal is nearly constant. The data are averaged from two experiments. Both within and between experiments no data points varied by more than 5%. (*Right*) After the reincubation as in the left panel cells were treated with PI-PLC at 0°C. The resulting supernatants were fractionated by SDS-PAGE and blotted to nitrocellulose. The blots were then probed with ^{125}I-streptavidin. As shown, the amount of released Thy-1 remains essentially unchanged. In three experiments, no progressive reduction in signal was seen. The band $-b$ corresponds to PI-PLC supernatants from cells which were not biotinylated. In the band *0*,− biotinylated cells were neither recultured nor treated with PI-PLC. The samples *0*, 15, 30, 60, *120 +PI-PLC* were recultured for the indicated number of minutes before PI-PLC treatment. The star designates Thy-1, whose identity was confirmed by immunoprecipitation; the arrowhead at the right designates the released protein of ~130 kD. TAP (~15 kD) is not detected. Arrowheads at the left are molecular mass standards (top-to-bottom: 93, 68, 31, 16 kD).

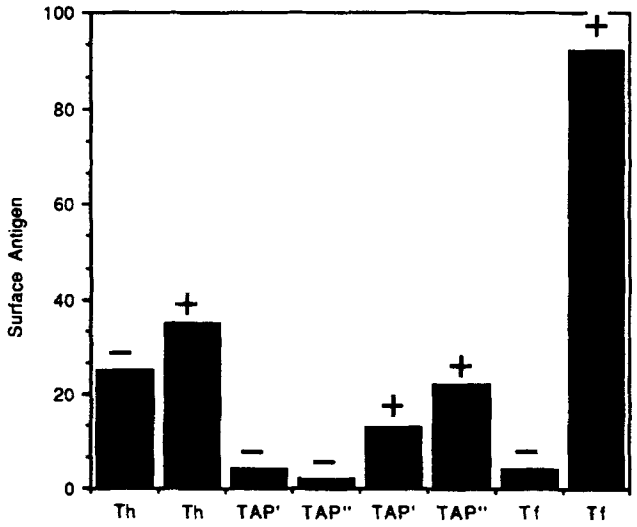

Figure 4. Flow-cytometric evaluation of the extent to which each antigen can be reexpressed on the cell surface after its removal by PI-PLC or protease treatment at 0°C. Control and enzyme-treated cells were stained at once (−) or recultured at 37°C for 1 h (+) before staining. The mean fluorescence of the control samples is in each case set equal to 100%. For TAP, antigen removal was either with PI-PLC (*TAP'*) or with Pronase (*TAP"*). *Th*, Thy-1; *Tf*, transferrin receptor. Extensive reexpression of the transferrin receptor, but not of the lipid-anchored proteins is observed. The amount of Thy-1 released by PI-PLC cannot be increased by a 10 times further increase in enzyme dose at 0 or 37°C.

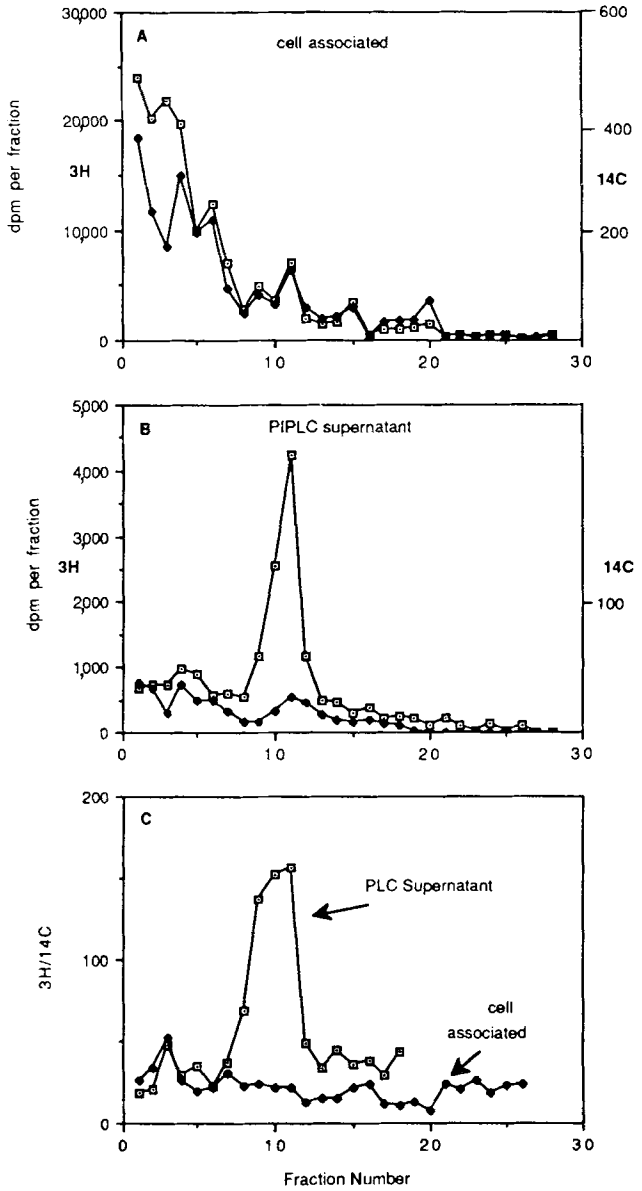

Figure 5. Evaluation of the turnover of Thy-1. Cells were pulse labeled with [^{14}C]glucosamine or pulsed with [^{3}H]glucosamine and chase incubated for 2 d. The two samples were then combined and lipid-anchored proteins were released by PI-PLC treatment at 0°C and analyzed by SDS-PAGE. Gel slices were counted for ^{3}H and ^{14}C. The raw data (*A* and *B*) and isotope ratios (*C*) are given. Note the uniquely high isotope ratio for Thy-1, whose identity was confirmed in independent immunoprecipitation experiments using both labeled sugars and ethanolamine (14). In the present experiments, the incorporation of labeled sugars was not sufficiently intense to detect TAP, which contains *O*-glycans, but not *N*-glycans. In *A* and *B*, ^{3}H is ⊡ and ^{14}C is ◆.

capped and internalized after antibody cross-linking (2, 24, 55, 59). The significance of the documented slow diacytosis of 5'-nucleotidase (56, 61, 63) is not clear since this enzyme is only partially sensitive to PI-PLC (32).

The double-label protocol used to evaluate turnover of PI-PLC sensitive cell-surface Thy-1 clearly makes Thy-1 stand out by contrast to the average glycoprotein. Thus, the *N*-glycans of this protein are unusually stable and we infer that its polypeptide must be at least equally stable. Earlier studies of Thy-1 turnover (57) have not focused on PI-PLC-sensitive molecules and are, unfortunately, incompletely described. Studies of 5'-nucleotidase turnover (3) are flawed by their dependence on the prolonged use of inhibitors of protein synthesis.

If lipid anchoring of a surface antigen allows it to avoid coated pits and be spared from degradation, as is suggested by the present data, one might also expect that these proteins would move unimpeded from their site of synthesis to the plasma membrane. This notion is supported by (*a*) their high concentration at the cell surface and (*b*) the observation that mutant lymphomas in which Thy-1 retains its short cytoplasmic segment do not transport Thy-1 to their surface (10, 15).

Thus, the present observations point to several further considerations that may explain the existence of glycolipid-anchoring of proteins such as Thy-1.

One is economy of biosynthetic effort, especially for antigens on highly endocytic cell surfaces, i.e., endocytosis of such proteins via coated pits might result in their degradation. A second consideration is that their frequent diacytosis, which would expose them to endosomal acidity, might denature critical epitopes.

The extended half-life of Thy-1, and possibly other lipid-anchored proteins, may explain why this protein is abundant at the surface of nucleated cells and why several other lipid-anchored proteins are even retained at the surface of red blood cells. It also implies that cells must develop accessory mechanisms for causing turnover of lipid-anchored proteins, for example, mobilization and/or activation of anchor-hydrolytic enzymes.

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