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Spontaneous incorporation of the glycosyl-phosphatidylinositol-linked protein Thy-1 into cell membranes

(GPI-linked protein/fluorescence recovery after photobleaching/lateral mobility/lymphocyte)

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ABSTRACT **Thy-1 is a membrane protein that is attached to the plasma membrane by a glycosyl-phosphatidylinositol anchor. Purified rat brain Thy-1 could be reincorporated into the plasma membrane of murine Thy-1$^-$ cells directly from aqueous suspension and without the use of detergents. A peripheral staining pattern similar to that observed for endogenous Thy-1 was achieved. Treatment with phosphatidylinositol-specific phospholipase C removed nearly all antibody staining due to either endogenous or inserted Thy-1. Fluorescence recovery after photobleaching (FRAP) was used to compare the lateral mobility of endogenous and inserted Thy-1. Both forms exhibited large lateral diffusion coefficients, but with a substantial immobile fraction (≈50%) indicating that the immobile fraction was not due either to chemical differences between inserted and native Thy-1 or to some surface Thy-1 molecules having a protein anchor. However, the inserted Thy-1 failed to activate mouse T lymphocytes upon crosslinking as assayed by [^{3}H]thymidine uptake. Since Thy-1 could be directly labeled with rhodamine, the effect of the size of the labeling ligand on the mobility obtained by the FRAP technique could be explored. Rhodamine-conjugated MRC-OX7 monoclonal antibody or its fragments [R-F(ab)$_2$ or R-Fab] were compared with rhodamine as labels for Thy-1. The measured diffusion coefficients were 1.6×10^{-9}, 2.0×10^{-9}, and 3.2×10^{-9} cm^2/sec for Thy-1 labeled with R-F(ab)$_2$, R-Fab, and rhodamine, respectively; mobile fractions were all in the 40–50% range. Thus, the size of the ligand affects the lateral mobility of this labeled membrane protein to a measurable extent.**

Thy-1, a member of the immunoglobulin superfamily, is a cell surface differentiation antigen present in rodent thymocytes and brain cells (1). It has been shown recently that Thy-1 mediates adhesion of mouse thymocytes to thymic epithelial cells through a Ca^{2+}-independent mechanism (2), as well as an inhibitory interaction between neurites and mature astrocytes (3). Mature Thy-1 is a small glycoprotein of 111 amino acids that is attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor covalently linked to the carboxyl-terminal amino acid (4). Phospholipid analogues have been inserted into plasma membranes for studies on lateral mobility (5), and it was of interest to undertake similar studies for a GPI-linked glycoprotein. Low levels of the GPI-linked glycoprotein decay-accelerating factor (DAF) have been inserted into erythrocytes by incubation of cells with the pure molecule (6), and Thy-1 has been incorporated into mouse T lymphocytes at high levels in the presence of polyethylene glycol (PEG) (7). In neither of these cases was the distribution or lateral mobility of the inserted molecule investigated. The membrane form of the variant surface glycoprotein of *Trypanosoma brucei* has been reincorporated into cultured cells from detergent solution and retains the very low lateral mobility measured in the trypanosome coat (8). In the present study, rat brain Thy-1 has been incorporated into murine lymphoid cells and the inserted molecule has been compared with endogenous Thy-1 in terms of staining patterns, lateral mobility, phosphatidylinositol-specific phospholipase C (PIPLC) cleavability, and ability to act as a target for mitogenesis.

MATERIALS AND METHODS

Thy-1 Preparation and Labeling. Thy-1 was solubilized from rat brain with deoxycholate and purified as described (9). Detergent was removed by dialysis against NH_4HCO_3 to yield Thy-1 in the form of an oligomer containing about 16 Thy-1 molecules (10). The isolated Thy-1 was conjugated with Lissamine rhodamine B sulfonyl chloride (Molecular Probes) by a procedure modified from an antibody labeling protocol (11). Thy-1 solution (3 mg/ml) was adjusted to pH 9 with 1 M $NaHCO_3$ (pH 9.0) before the dye was added. Rhodamine was dissolved in acetone and added to Thy-1 in a single addition at room temperature to give a 30:1 molar ratio of rhodamine to protein and a final acetone concentration of about 0.4%. The reaction time was 3 min at room temperature. The rhodamine-conjugated Thy-1 (R-Thy-1) was then separated from free dye by passage of the reaction mixture through a Sephadex G-50 fine column (Pharmacia), followed by exhaustive dialysis; the final molar ratio of fluorochrome to protein was 0.2:1.

Preparation and Labeling of Antibodies and Their Fragments. MRC-OX7 mouse monoclonal antibody (IgG) against the Thy-1.1 determinant was used for all the studies. Fab fragments were prepared either from F(ab)$_2$ (12) or from intact IgG by papain digestion (11) at an enzyme/substrate ratio of 1:100 (wt/wt) at 37°C for 17 hr. Fab fragments were separated from the digestion mixture by chromatography on Sephacryl S-200. After PAGE, silver staining of the Fab preparation indicated that no intact IgG remained. Antibody or fragments were conjugated with Lissamine rhodamine B sulfonyl chloride as described above. The molar ratio of fluorochrome to protein in the conjugate did not exceed 2.0. The specificity of each antibody or fragment preparation was demonstrated by the absence of staining of both chicken embryo fibroblasts (Thy-1$^-$) and AKR1/G1M1 mouse lymphoma (Thy-1$^-$) cells.

Cells. AKR1/G1 (Thy-1$^+$) and AKR1/G1M1 (Thy-1$^-$) lymphoma cell lines were gifts from I. Trowbridge (Salk Institute, La Jolla, CA). Chicken embryo fibroblasts were obtained

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Abbreviations: FRAP, fluorescence recovery after photobleaching; GPI, glycosyl-phosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C; R-antibody or R-antigen, rhodamine-conjugated antibody or antigen; PBS(+), phosphate-buffered saline with Ca^{2+} and Mg^{2+}.

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from primary culture of 12-day chicken embryos. Mouse splenocytes were obtained from spleen of BALB/c mice. COS-1 cells were cultured as described (11). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete medium).

Thy-1 Incorporation. AKR1/G1M1 cells or mouse spleen cells were suspended at 10^6–10^7 cells per ml in complete medium. A small drop of suspended cells was placed on a coverslip (12 mm × 12 mm) and incubated for 20 min at 37°C, enabling 60–70% of the cells to attach to the glass substrate. Attached cells were placed upside down on a 20-μl droplet of Thy-1 (350 μg/ml) in phosphate-buffered saline with Ca^{2+} and Mg^{2+} [PBS(+)]. The cells were incubated in the Thy-1 solution for 20 min at 37°C and carefully washed twice in PBS(+). Cells were then incubated with antibody or its fragments for 10 min at room temperature. Coverslips were mounted onto a metal chamber slide in PBS(+) for microscopic examination and measurements of fluorescence recovery after photobleaching (FRAP). Thy-1 was inserted into COS cell cultures by a protocol identical to that used for attached AKR1/G1M1 cells.

Thy-1 was also incorporated into suspension cells. AKR1/G1M1 or mouse splenocytes in suspension (2–5 × 10^6 cells per ml) were pelleted and resuspended to 3 × 10^7 cells per ml in complete medium. Thy-1 (40 μl at 2.5 mg/ml) was added to 20 μl of cell suspension. This mixture was incubated at 37°C in CO_2 incubator for 20 min and washed three times with PBS(+).

Mitogenesis Assay. The assay was based on previous work (7) and modified slightly. Approximately 2 × 10^6 splenocytes from BALB/c mice into which rat brain Thy-1 had been inserted were incubated with appropriate anti-Thy-1 antibodies at 4°C for 30 min and then washed prior to incubation with secondary antibodies and addition of phorbol 12-myristate 13-acetate (25 ng/ml). Cells (≈2 × 10^5 per well) were plated in 96-well microtiter plates in complete medium (0.1 ml per well) and placed in a humidified CO_2 incubator at 37°C. Each well received 0.4 μCi (14.8 kBq) of [^{3}H]thymidine 4 hr before the end of incubation period, and the uptake of radioactivity was determined after harvesting onto glass-fiber filters.

PIPLC Assay. A coverslip with attached AKR1/G1M1 cells into which Thy-1 had been inserted was placed upside down on a 12-μl droplet of 0.01 unit of PIPLC (ICN) in Dulbecco's modified Eagle's medium with 1% fetal bovine serum. The cells were incubated for 30 min at 37°C, carefully washed twice in PBS(+), and then incubated with antibody

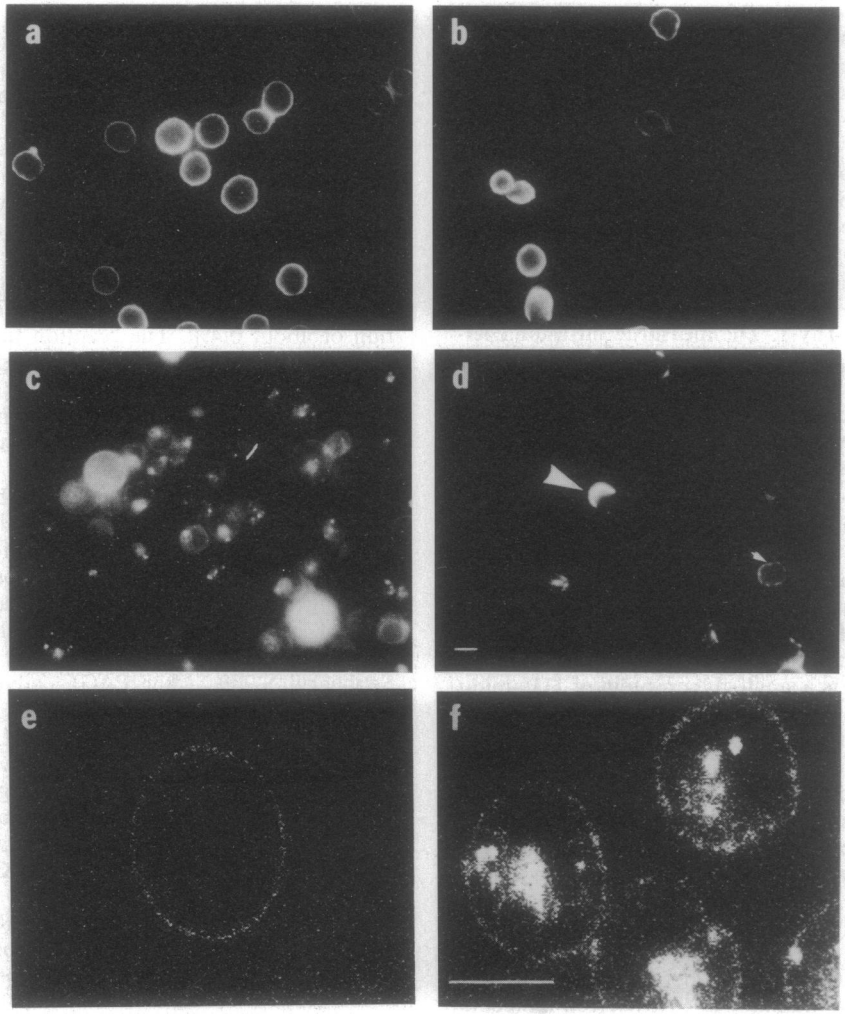

FIG. 1. Fluorescence staining of native and inserted Thy-1. (*a* and *b*) Native Thy-1 in AKR1/G1 (Thy-1$^+$) cells (*a*) and inserted Thy-1 in AKR1/G1M1 (Thy-1$^-$) cells (*b*) have similar staining patterns. Both cell samples were fixed before staining with R-F(ab)$_2$ fragment (60 μg/ml) of MRC-OX7. (*c*) AKR1/G1M1 cells were fixed after incubation with R-Thy-1 (360 μg/ml) for 20 min at 37°C; note that some internalization occurred (see text). (*d*) Patching (small arrow) and capping (large arrow) of inserted Thy-1 in AKR1/G1M1 cells. After Thy-1 insertion, the cells were incubated with MRC-OX7 F(ab)$_2$ (60 μg/ml) followed by fluorescein-labeled second antibody. The cells were photographed after 30 min to demonstrate patching and capping over time. (*e* and *f*) Confocal images of inserted R-Thy-1 near midsection (*e*) and near dorsal surface (*f*) of AKR1/G1M1 cells with the microscope set at full confocality (smallest emission pinhole setting). (Bars = 10 μm.)

or its fragments for 10 min at room temperature. Coverslips were mounted onto a metal chamber slide in PBS(+) for microscopic examination.

Fluorescence Microscopy. Fluorescence images were taken either with a Zeiss IM35 or a Nikon Optiphot-2 microscope using phase contrast and rhodamine optics. Identical exposures and development times were employed for the micrographs in each figure. For fixation, cells were incubated with 3.6% formaldehyde in PBS(+) for 15 min at room temperature. Cells were then washed extensively with PBS(+) before antibody labeling.

Confocal Microscopy. Confocal images were acquired on the Bio-Rad MRC-600 microscope using rhodamine optics and at nearly full confocality.

FRAP. Measurements were made at room temperature employing a ×40 oil objective (n.a., 1.3), and recovery was recorded as described (13).

RESULTS AND DISCUSSION

Insertion of Thy-1. Thy-1 incorporation into lymphocytes was initially attempted by incubation in the presence of 10–20% polyethylene glycol (PEG), since this had yielded stable incorporation of 100,000–500,000 Thy-1 molecules per cell (7). However, in the present study, Thy-1 incorporated in this way yielded a punctate staining pattern and no fluorescence recovery was seen after photobleaching.

Insertion of Thy-1 by incubation in buffered salt solution yielded about 40,000 molecules per cell on lymphocytes (7), and when this procedure was tried with AKR1/G1M1 (Thy-1$^-$) cells, a peripheral staining pattern (Fig. 1*b*) was seen similar to that observed for native Thy-1 on AKR1/G1 (Thy-1$^+$) cells (Fig. 1*a*). Addition of secondary antibody caused prominent patching and capping of the inserted Thy-1 (Fig. 1*d*), similar to that observed with native Thy-1 in AKR1/G1 cells (data not shown). Thus, with respect to the initial distribution and antibody-induced aggregation, native and inserted Thy-1 behaved similarly.

When Thy-1 directly labeled with rhodamine (R-Thy-1) was incorporated into AKR1/G1M1 cells, the distribution pattern (Fig. 1*c*) was similar to that seen with the Thy-1 labeled with antibody, although the staining pattern was consistently fainter with some aggregation and internalization visible in some of the cells. This view was extended using confocal microscopy of R-Thy-1 inserted into AKR1/G1M1 cells. Aggregates and/or internalized materials appeared near the poles of the cells—either the substrate attached end or the free hanging "pole" (Fig. 1*f*). Near the midsection of the cell "ring," staining was observed with no internalized material visible (Fig. 1*e*). It is curious that Thy-1 seems aggregated at cell–cell or cell–substrate contacts. The nature and functionality of these aggregates are not clear. Several experiments indicated that AKR1/G1M1 cells were capable of internalization under the conditions employed for Thy-1 insertion. First, without any Thy-1 insertion, R-Fab fragments of MRC-OX7 antibodies incubated with AKR1/G1M1 cells were internalized to a limited extent after 10 min at room temperature without any staining of the plasma membrane. Second, when antibody staining of Thy-1 inserted into AKR1/G1M1 cells was done on ice, internalized staining was much less apparent (data not shown).

PIPLC digestion of the inserted material was employed as a further check of correct insertion. The reasoning was that release of the Thy-1 ectodomain would probably result only if the GPI anchor of Thy-1 were correctly intercalated into the bilayer. Indeed, PIPLC treatment resulted in nearly complete loss of fluorescence (Fig. 2), indicating that the inserted Thy-1 could be cleaved by PIPLC. Cleavage of endogenous Thy-1 in AKR1/G1 cells by PIPLC was also almost complete (data not shown).

In addition to using PEG as an agent to facilitate insertion of Thy-1 into cells, we also tested Pluronic detergent (BASF

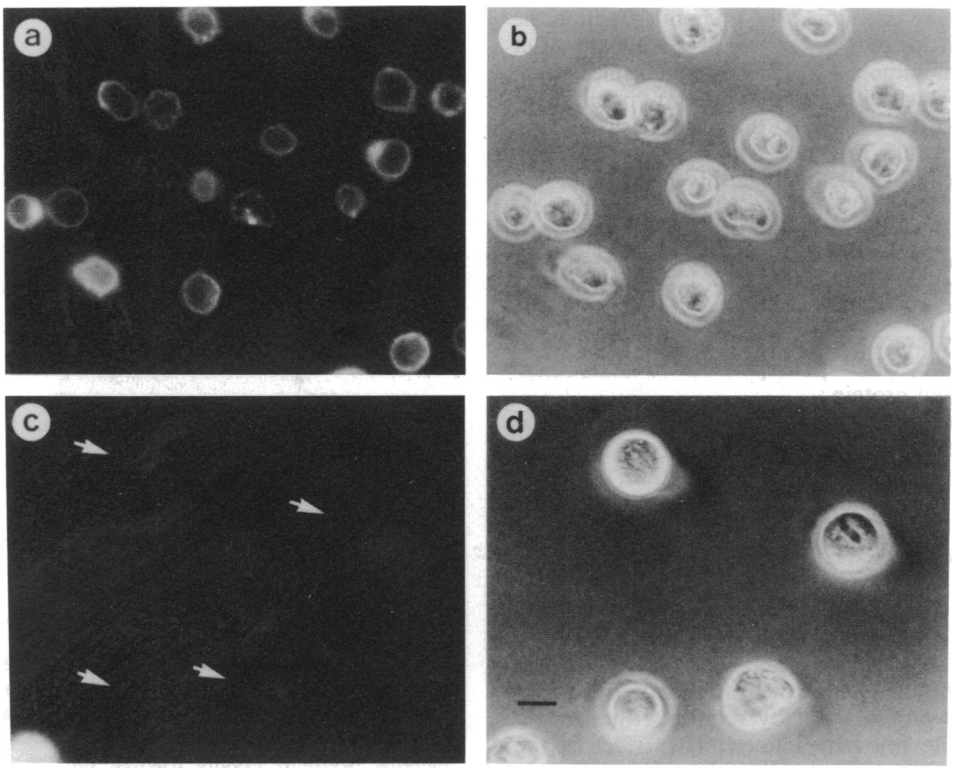

FIG. 2. PIPLC treatment of the inserted Thy-1. (*a*) Fluorescence image of inserted Thy-1 in AKR1/G1M1 cells that were stained with R-F(ab) fragment (60 μg/ml) of MRC-OX7 at 4°C to minimize internalization. (*b*) Phase-contrast image of same cells as in *a*. (*c*) PIPLC treatment removed most of fluorescence staining of inserted Thy-1 in AKR1/G1M1 cells; arrows show positions of cells as obtained from companion phase-contrast image (*d*) of cells in *c*. (Bar = 10 μm.)

Table 1. Mobility of endogenous and inserted Thy-1

Cells	Mode of expression of Thy-1	D, (cm^2/sec) × 10^9	Mobile fraction, %	Number of measurements
AKR1/G1	Endogenous	2.0 ± 0.6	45 ± 10	22
AKR1/G1M1	Inserted	2.1 ± 0.5	46 ± 10	32
CEF*	Inserted	1.6 ± 0.5	49 ± 8	20
COS-1	Transfected (17)	2.7 ± 1.0	55 ± 10	32
COS-1	Inserted	1.9 ± 0.6	45 ± 12	16

Cells were labeled with MRC-OX7 Fab fragments (100 μg/ml). D, mobile fraction (mean ± SD).
*CEF, chicken embryo fibroblasts.

Wyandotte, Wyandotte, MI). Cells were incubated with Thy-1 plus the detergent at low levels (5–100 μg/ml); a diffuse, peripheral staining pattern was obtained after labeling the cells with antibody. However, most cells "blebbed" within 15 min of treatment with the Pluronic detergent, and it was concluded that incorporation of Thy-1 in the presence of agents that give membrane destabilization was not satisfactory for studies on lateral mobility.

Lateral Mobility of Inserted Thy-1. The inserted Thy-1 was laterally mobile, and the diffusion coefficients and mobile fractions were similar for inserted and native Thy-1 in lymphoma cells (Table 1). In addition, the mobility of Thy-1 inserted into COS-1 cells was similar to the mobility of Thy-1 transiently expressed in transfected COS-1 cells (Table 1). Thus, native and inserted Thy-1 exhibited similar lateral mobility. It is especially curious that the mobile fraction of inserted Thy-1 was approximately that of native Thy-1. The substantial immobile fraction of Thy-1 and other GPI-linked proteins is an unexpected and unexplained characteristic of these proteins. Although it could be argued that the immobile fractions are photochemical artifacts induced by photobleaching, it is also significant that similar immobile fractions were obtained whether directly labeled Thy-1 or antibody-labeled Thy-1 was measured (Fig. 3). One might expect that if photobleaching were producing the immobile fraction the photochemical environments of the two probes would differ enough that different immobile fractions would be measured. Furthermore, other work suggests that different populations of endogenous Thy-1 exist. The fact that 10–20% of Thy-1 in T-lymphoma cells could not be solubilized by nonionic detergent was taken to indicate that this fraction of the antigen is a constituent of a heteropolymeric complex of detergent-resistant membrane proteins (14). In addition, it was recently shown that GPI-linked surface molecules, including Thy-1, were associated with the cytoplasmic protein tyrosine kinase $p56^{lck}$ (15), indicating that such GPI-linked proteins indirectly interact with proteins on the inner face of the plasma membrane, which perhaps leads to immobilization of the lipid-linked protein.

This work also shows that the immobile fraction is not due to chemical differences between the reinserted rat brain Thy-1 and the endogenous murine Thy-1. Further, our work demonstrates that the immobile fraction of native Thy-1 could not be due to Thy-1 with a protein anchor, since all of the reinserted antigen, which has a similar immobile fraction as native Thy-1, is GPI-linked. These results support other data showing that the mature glycoprotein is exclusively GPI-linked (16).

Effect of Size of the Labeling Ligand on the Measured Lateral Mobility. Photobleaching measurements of the mobility of membrane proteins usually require a fluorescent antibody to label the relevant antigen. The effect of the antibody label has been a long-standing concern in measurements of membrane protein lateral mobility by the FRAP technique. The ability to insert a directly conjugated Thy-1 into the membrane allowed a comparison of the lateral mobility of R-Thy-1 with that of antibody-labeled Thy-1 to be made. Fig. 3 shows the mobility of inserted Thy-1 as a function of labeling ligand size employing R-IgG, R-F(ab)$_2$, R-Fab, and rhodamine as probes. Particularly in the case of R-Thy-1, the laser beam was positioned to avoid aggregated and/or internalized material. A gradual, monotonic increase in the lateral diffusion coefficients was observed as the size of the labeling ligand was decreased from R-IgG to rhoda-

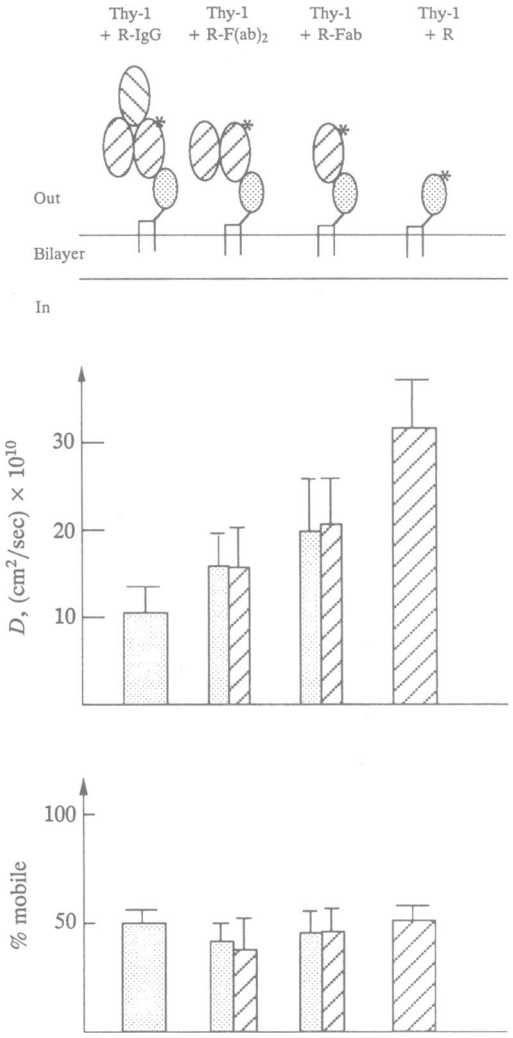

FIG. 3. Effect of labeling ligand size on lateral mobility of Thy-1 in AKR1/G1 and AKR1/G1M1. (*Top*) Schematic of various ligands (hatched ovals) bound to Thy-1 (stippled oval); *, R, rhodamine. (*Middle*) Diffusion coefficient for the various ligand/Thy-1 combinations. (*Bottom*) Mobile fraction for the various ligand/Thy-1 combinations (hatched bars, inserted Thy-1; stippled bars, endogenous Thy-1). Antibodies or their fragments were used at the following nominal concentrations: 60 μg/ml (IgG), 80 μg/ml [F(ab)$_2$], and 100 μg/ml (Fab). Inserted Thy-1 labeled with IgG yielded no mobile Thy-1 in repeated attempts. The reasons for this are unknown.

mine. Mobile fractions remained in the range of 40–60% for all ligands measured. In the case of Thy-1, at least, the Fab fragment used for FRAP reduced the measured diffusion coefficient to about two-thirds of the unliganded value. This effect of ligand size can be contrasted to a recent study (17) showing no correlation between ectodomain size and lateral mobility for a series of unrelated proteins.

Inserted Thy-1 as a Target for Mitogenesis. Although the results above suggested that inserted Thy-1 was intercalated into the lipid bilayer via its GPI anchor, the functionality of this insertion remained in question. It was therefore of interest to determine whether this Thy-1 could function as a target for mitogenesis, as is the case for endogenous Thy-1. Anti-Thy-1 antibodies are generally mitogenic for mouse T cells if the antibody is crosslinked with an anti-immunoglobulin second antibody and the cells are cultured in the presence of phorbol myristate acetate (18). Rat brain Thy-1 (Thy-1.1 determinant) was inserted into mouse lymphocytes carrying the Thy-1.2 allotype and mitogenesis was tested with the MRC-OX7 (anti-Thy-1.1) antibody as well as an antibody against Thy-1.2. The mitogenic effect was seen with the endogenous Thy-1 but not with the inserted molecule (data not shown). This negative result had also been observed in the experiments where Thy-1 was inserted in the presence of PEG (7).

The reasons for the failure of inserted Thy-1 to act as a target for mitogenesis are unclear and are in contrast to studies where Thy-1 was introduced by transfection into human Jurkat cells and functioned as a target for signal transduction (19). It is possible that some structural difference between inserted and endogenous Thy-1 may account for the functional activity. For example, it could be that some oxidation of the lipid anchor has occurred in the purification of Thy-1. Alternatively, tissue-specific posttranslational modifications of the molecule may be important. The carbohydrate structures of brain Thy-1 are different from those of the lymphoid forms of the molecule (20), and there are differences in some carbohydrates attached to the backbone of the GPI anchor (4). These structural differences may lead to a failure of inserted Thy-1 to appropriately enter a signal-transduction pathway, such as those mediated by protein kinases (15).

The current data show that Thy-1 can be inserted into cells in a manner that mimics the biophysical properties of endogenous Thy-1, and this system should be of further interest in studies on membrane dynamics and domain structure (21). However, the requirements for functional insertion remain to be established.

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