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# The Thy-1 antigen exhibits rapid lateral diffusion in the plasma membrane of rodent lymphoid cells and fibroblasts

**(fluorescence recovery after photobleaching)**

AKIRA ISHIHARA*, YU HOU*, AND KEN JACOBSON*†

Laboratories for Cell Biology, *Department of Anatomy and †Cancer Research Center, School of Medicine, University of North Carolina, Chapel Hill, NC 27514

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ABSTRACT **Thy-1 is a plasma membrane protein, but its primary structure lacks the typical membrane-spanning sequence. Recent studies revealed that a glycophospholipid is covalently bound to the carboxyl terminus, suggesting that the protein is integrated into the plasma membrane by this lipid moiety. Lateral diffusion of Thy-1 was measured in mouse thymocytes, lymphoma cells, and fibroblasts by the fluorescence recovery after photobleaching technique. Thy-1 was labeled with rhodamine-conjugated anti-Thy-1 monoclonal antibodies. Diffusion coefficients of 2–4 × $10^{-9}$ $cm^2$/sec were obtained for the antigen–antibody complex in all the cell types. About 50% of the Thy-1 was mobile. The diffusion coefficient for the mobile fraction of Thy-1 is considerably larger than the diffusion coefficients of many other plasma membrane proteins. Rather, the diffusion coefficient of Thy-1 is similar to those of lipid analogs embedded in the same membrane, providing strong support for the suggested lipid anchoring of this antigen.**

Lateral diffusion has been measured for various membrane proteins of the plasma membrane of animal cells (1–3). In most but not all cases, proteins have been found to diffuse more slowly than expected based on protein diffusion in artificial bilayers, suggesting that interactions between the diffusant and other membrane proteins and/or peripheral structures retard lateral mobility (2). Thy-1 is a membrane glycoprotein that is present in copious amounts in rodent thymocytes and brain cells (4). Since its primary sequence of 111 residues lacks hydrophobic sequences typical of membrane-spanning proteins, it has been suggested that this antigen is integrated in the bilayer by a nonpeptide protein moiety (4). Very recent studies (5, 6) have revealed that, in fact, a glycophospholipid is covalently bound to the carboxyl terminus of Thy-1. The protein appears to be anchored in the plasma membrane bilayer by this lipid tail. Although the lateral diffusion of Thy-1 had been measured (7, 8), the two results yielded different diffusion coefficients. In light of the striking new structural data and the previous discrepancy in lateral diffusion results, we measured the lateral diffusion coefficients of Thy-1 in thymocytes, lymphoma cells, and fibroblasts by the fluorescence recovery after photobleaching (FRAP) technique using fluorescent monoclonal antibodies to tag the antigen. The diffusion coefficient for Thy-1 is >10 times greater than those measured for many other plasma membrane proteins studied in the past; its diffusion coefficient is comparable to those of lipid analogs embedded in the membrane. The rapid diffusion of Thy-1 is consistent with putative lipid anchoring of the glycoprotein to the plasma membrane bilayer.

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## MATERIALS AND METHODS

**Antibodies.** A rat monoclonal antibody (IgG) against Thy-1 (T24/40) was provided by I. Trowbridge (Salk Institute, San Diego, CA). Antibody in ascites fluid was purified by ammonium sulfate precipitation and conjugated with Lissamine rhodamine B sulfonyl chloride (Molecular Probes, Junction City, OR) as follows: 1 part of 1 M $NaHCO_3$ (pH 9.0) was added to 4 parts of phosphate-buffered saline (PBS; 140 mM NaCl/2.7 mM KCl/8.1 mM $Na_2HPO_4$/1.5 mM $KH_2PO_4$/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$, pH 7.4) containing the antibody at a concentration of ≈1 mg/ml. The rhodamine was dissolved in 99% acetone and added slowly to the antibody; the final ratio of rhodamine to protein was 50:1 (mol/mol), and the final acetone concentration was <0.4%. The mixture was incubated at room temperature for 20 min and centrifuged for 5 min to remove insoluble material. The antibody was separated from free dye by passing the mixture through a Pharmacia Sepharose G-50 fine column and eluting with PBS. The antibody was dialyzed against PBS with a few grams of Dowex 2-X8 per liter in the dialysis fluid for ≈36 hr to ensure removal of any unconjugated dye. The molar ratio of fluorochrome/protein ranged from 2.1:1 to 4.1:1.

**Cells.** Thymocytes were isolated from thymus of 6-week-old mice. Lymphoma cell lines [AKR1/G1 (Thy-$1^+$), AKR1/G1M1 (Thy-$1^-$)] were generous gifts from I. Trowbridge, and they were grown in Dulbecco's minimal essential medium supplemented with 10% horse serum. Mouse fibroblasts, C3H/10T1/2 and BALB/3T3, were grown on coverslips in Eagle's basal medium with Earle's salts (GIBCO) plus 5–10% fetal bovine serum and Dulbecco's minimal essential medium with 10% fetal bovine serum, respectively. BG9 human fibroblasts were cultured on coverslips in Eagle's minimal essential medium with Earle's salts plus 10% fetal bovine serum. To immobilize lymphoma cells and thymocytes on coverslips, these cells were washed twice in PBS and deposited on coverslips pretreated with 0.1% poly-D-lysine.

Cells were stained with 20–60 μg of the conjugated anti-Thy-1 antibody per ml and rinsed with PBS. They were also stained with 1-acyl-2(*N*-4-nitrobenzo-2-oxa-1,3-diazole aminocaproyl) phosphatidylcholine (NBD PtdCho; Avanti Polar Lipids), which was dissolved in 99% ethanol, diluted in PBS to 4 nM, and sonicated at 4°C.

**Fluorescence Recovery After Photobleaching (FRAP).** Measurement of diffusion coefficients by FRAP was essentially as described (9). Briefly, this version of the method is based on photobleaching a small circular region on the surface of single cells bearing fluorescent molecules to destroy the emission from that region. Subsequently, the recovery of fluorescence

Abbreviations: FRAP, fluorescence recovery after photobleaching; diI-$C_{16}$(3), 3,3'-dihexadecylindocarbocyanine; NBD PtdCho, 1-acyl-2(*N*-4-nitrobenzo-2-oxa-1,3-diazole aminocaproyl) phosphatidylcholine.

due to the diffusion or flow of unbleached fluorophores from the surrounding area into the irradiated area is measured. When only isotropic lateral diffusion occurs, the recovery kinetics, characterized by the time, $t_{1/2}$, to obtain 50% of full recovery, are related to $D$, the diffusion coefficient by $D = W_s^2 \gamma / 4t_{1/2}$, where $W_s$ is the $e^{-2}$ radius of the Gaussian profile laser beam used for both photobleaching and measuring fluorescence, and $\gamma$ is a parameter that depends on the extent of photobleaching (10). The mobile fraction of the measured population of probe molecules is obtained from the degree to which the final fluorescence level approaches the pre-bleach fluorescence value.

FRAP measurements were made with the ×40 oil objective (n.a., 1.3) on the photobleaching microscope; the focussed Gaussian laser beam radius was calculated to be ≈1 μm, and this calculation was confirmed by quantitative fluorescence microscopy using digital image processing. In this measurement, the focussed beam was imaged by the fluorescence excited from a thin film of 3,3'-dihexadecylindocarbocyanine [diI-$C_{16}$(3); see ref. 11] and its $e^{-2}$ width was obtained directly from the beam intensity profile taken from the digitized image (12). To measure the rapid ($t_{1/2}$, <1 sec) fluorescence recoveries of Thy-1, a photon counter (Thorn EMI GenCom, Fairfield, NJ) coupled to a multichannel analyzer (Nucleus, Oak Ridge, TN) was used. Alternatively, analog detection using an amplifier having a time constant of ≈20 msec and a storage oscilloscope were used to record the fluorescence recovery kinetics. Bleach times were from 37 to 150 msec; bleach powers (at the laser) were from 8 to 200 mW at 515 nm. The bleaching beam was attenuated by 100-fold or 1000-fold for the recovery measurement. For the fibroblasts, a 350-μm pinhole in an intermediate image plane was used to limit the collection depth of the photomultiplier. A 200-μm pinhole was used to discriminate surfaces in the round thymocytes and lymphoma cells. The measurements were done at room temperature.

**Antibody Dissociation.** The dissociation rate of the labeled antibody was estimated by measuring the decrease in fluorescence intensity of stained thymocytes at various times after labeling. The fluorescence intensity was measured with a digitized fluorescence microscope, as described elsewhere (12). Fluorescent images of given thymocytes were taken at various times, and the area average fluorescence intensity over that cell was obtained as a gray level average of the pixels in the cell area. Corrections for fluorescence fading, although very small, were made.

## RESULTS

A rat monoclonal antibody (IgG) against murine Thy-1 was conjugated with rhodamine. The specificity of the labeled antibody was demonstrated by the lack of staining of BG-9 human fibroblasts and of a mutant Thy-1$^-$ lymphoma line (AKR1/G1M1). Mouse thymocytes, Thy-1$^+$ lymphoma (AKR1/G1), and fibroblasts (C3H/10T1/2, BALB/3T3) were stained diffusely, indicating that the antibody did not induce crosslinking of the antigen. The antigens could be patched by addition of a secondary anti-rat IgG antibody. Almost all of the Thy-1 could be patched 1 hr after staining with the monoclonal antibody, suggesting that most of the antigen remained on the cell surface during the 1-hr period. To exclude the possibility that surface hopping of the antibody accounts for the FRAP, the dissociation rate of the antibody was estimated by measuring the decrease in fluorescence intensity of thymocytes at various times after labeling. The half-time for dissociation was 230 min; thus, only 0.3% or less of the bound antibody was estimated to dissociate within 6 sec, at which time the fluorescence recoveries were largely completed.

Lateral diffusion of the Thy-1 was measured by the FRAP technique as described (9). As shown in Table 1, the measurements yielded a diffusion coefficient of 2–4 × $10^{-9}$ $cm^2$/sec. All cell types gave similar values, although the density of Thy-1 on fibroblasts was considerably less than on thymocytes and lymphoma cells. A photometric measurement by quantitative digitized fluorescence microscopy (11) showed that the fluorescence per unit area in fibroblasts was ≈1/10th that in thymocytes. The mobile fractions ranged from 40% to 60% in all the cell types.

In addition, the diffusion coefficient of Thy-1 was compared to those for GP80, an 80-kDa integral membrane glycoprotein (13), diI-$C_{16}$(3), a lipid probe, and NBD PtdCho, a phospholipid analog (Fig. 1*A*). All these measurements were done in the same cell type, C3H/10T1/2 mouse fibroblasts; the diffusion coefficients for diI-$C_{16}$(3) and GP80 have been published (9). GP80, a putative membrane-spanning glycoprotein, has a small $D$ value (1–2 × $10^{-10}$ $cm^2$/sec) typical of many membrane proteins, while the $D$ value for diI-$C_{16}$(3) was ≈100 times greater. $D$ for Thy-1 was nearly the same as that for NBD PtdCho and almost 40 times larger than $D$ for GP80. The diffusion coefficient obtained for NBD PtdCho was consistent with previous results (14). The corresponding mobile fractions for each diffusant are presented in Fig. 1*B*. GP80 has a mobile fraction of ≈75% compared to ≈50% for Thy-1, while the two lipid probes have mobile fractions approaching 100%.

## DISCUSSION

The lateral diffusion of Thy-1 was measured in three different cell types by FRAP, and relatively large diffusion coefficients (2–4 × $10^{-9}$ $cm^2$/sec) were obtained for all the cell types. Two measurements of Thy-1 lateral diffusion in lymphocytes have been reported previously, but the results did not agree. Dragsten *et al.* (7) reported slow ($D \approx 10^{-10}$ $cm^2$/sec) diffusion, but the polyclonal rabbit anti-mouse brain antibody used to label the antigen was not as well characterized as the monoclonal anti-Thy-1 antibody used in this study. On the other hand, Woda and Gilman (8) reported considerably greater diffusion coefficients for Thy-1 using Fab fragments of the monoclonal antibody MRC OX-7. However, their measurements could be criticized on two counts. First, a parallel determination of the diffusion coefficient for surface immunoglobulin was larger than those reported for this protein in three previous studies by a factor of ≈3 (refs. 5, 16), calling into question the measurement of rapid Thy-1 diffusion. Second, the dissociation rate of this antibody fragment, which may have been very rapid (17), was not measured. Such dissociation could have led to an artificially high value of the diffusion coefficient. Thus, in light of the striking new structural data and the discordant previous diffusion results, we believed a more extensive study was warranted.

The result for Thy-1 diffusion is in striking contrast to the many previous measurements of sluggish glycoprotein lateral diffusion ($0.5 \times 10^{-10} \leq D \leq 6 \times 10^{-10}$ $cm^2$/sec) for plasma membrane proteins (1). Rather, the value is similar to those

Table 1. Diffusion coefficients and mobile fractions of Thy-1

| Cell type | Line | $D$ (×$10^{-9}$ $cm^2$/sec) | Mobile fraction, % |
|---|---|---|---|
| Fibroblast | C3H/10T1/2 | 3.9 (±0.3) | 46 (±4) |
| Fibroblast | BALB/3T3 | 3.1 (±0.4) | 45 (±9) |
| Thymocyte | * | 2.8 (±0.4) | 55 (±6) |
| Lymphoma | AKR1/G1 | 2.1 (±0.4) | 43 (±4) |

Diffusion coefficients and mobile fractions of the Thy-1 antigen were measured by FRAP in various cell types. Values in parentheses represent SEM.
*Isolated from mouse thymus.

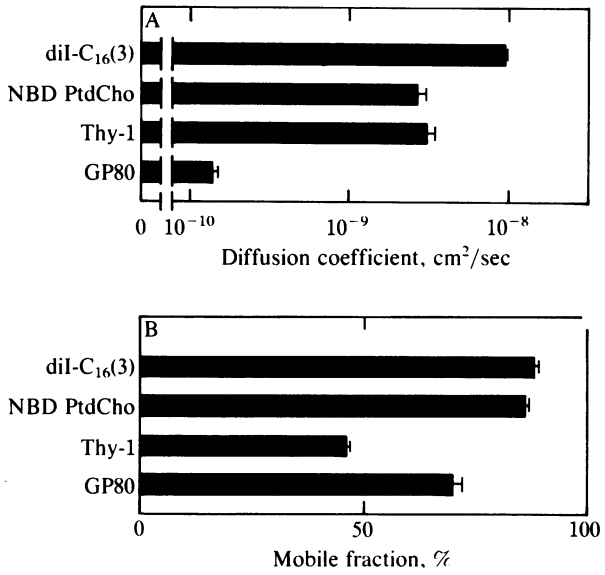


FIG. 1. Comparison of the Thy-1 diffusion coefficient (*A*) and mobile fraction (*B*) with those of other molecules. diI-$C_{16}$(3) and NBD PtdCho are fluorescent lipid analogs. GP80 is a putative transmembrane glycoprotein. The diffusion coefficients and mobile fraction for these molecules in C3H/10T1/2 cells were measured by FRAP. Error bars represent SEM.

of lipid probes such as diI-$C_{16}$(3) or NBD PtdCho. The relatively large diffusion coefficient measured for Thy-1 probably reflects the recently proposed structure for the molecule (5, 6) in which the glycoprotein is postulated to be integral by virtue of a glycophospholipid tail and not by the more typical hydrophobic membrane-spanning peptide. Assuming that this lipid tail is not associated with another membrane protein, diffusion of the lipid-linked glycoprotein would not be expected to be limited by membrane-associated cytoskeletal structure, as is the case, for example, with band 3 of the erythrocyte (18, 19). Its diffusion coefficient presumably is determined mainly by those factors that affect lipid diffusion, such as lipid composition and integral protein concentration (2, 20). It is of interest that a recent determination of the lateral mobility of another lipid-linked membrane protein, decay accelerating factor (21), also yielded large values of the diffusion coefficient ($\gtrsim 10^{-9}$ cm$^2$/sec; see ref. 22).

Since the lipid composition of viral envelopes reflects the lipid composition of the host cell plasma membrane (23), the lipid-linked structure and lipid-like lateral diffusion of Thy-1 may explain the observation that, of several major cell membrane glycoproteins, only Thy-1 is found in the envelopes of budding murine leukemia and vesicular stomatitis viruses (24). In addition, the rapid lateral diffusion of Thy-1 may play a role in the quick increase in intracellular $Ca^{2+}$ following antibody-mediated crosslinking of Thy-1 on lymphocytes (25).

The fact that the Thy-1 diffusion closely approaches that of the lipid probes indicates that the size of the extracellular ligand (i.e., the glycoprotein plus bound monoclonal antibody) and its possible interaction with the apposed extracellular matrix does not appreciably retard diffusion. Earlier measurements in artificial bilayers showed that the rate of lipid diffusion was not hindered by the presence of antibody bound to a haptenic group covalently linked to the lipid head group (26). Our findings suggest a clearance of 40 to 140 Å between bilayer surface and the bulk of the extracellular matrix structure. The minimum clearance (40 Å) is estimated by assuming that the 25-kDa glycoprotein moiety of Thy-1 is spherical, having a diameter of ≈40 Å, and the bound antibody lays nearly flat on the membrane bilayer surface. The maximum clearance (140 Å) is estimated by assuming that the antibody binding site is located on top of the glycoprotein, allowing the IgG to extend maximally ≈100 Å further into the extracellular medium.

One contrast to the diffusion of lipid analogs in artificial bilayers and in fibroblasts and to the diffusion of gangliosides (27) in fibroblast membranes is that a substantial fraction of the Thy-1 antigen was immobile. A substantial immobile fraction was also measured for the lipid-linked decay accelerating factor (22). An immobile fraction has been measured for lipid analog diffusion in egg membranes (28) and has been quite generally observed for plasma membrane glycoproteins (1). Several possibilities, not mutually exclusive, exist to explain this immobile fraction of Thy-1. (*i*) This fraction of the Thy-1 may be functionally immobilized by being "trapped" in a lipid domain or bound to another membrane protein that itself is immobilized via interaction with peripheral structures. (*ii*) A fraction of the antigen may be sterically trapped or specifically anchored in certain local areas where the clearance between the membrane and the extracellular matrix is smaller. (*iii*) The immobile fraction may reflect the claim that at least part of the Thy-1 population is anchored by a hydrophobic peptide transmembrane segment (29). Some recent biochemical results bear on these possible causes of the immobile fraction. The observation that 10–20% of Thy-1 in T-lymphoma cells cannot be solubilized by nonionic detergent was taken to suggest that this fraction of the antigen is a constituent of a heteropolymeric complex of detergent-resistant membrane proteins (30); this observation supports any or all of the above possibilities. In addition, Low and Kincade (5) reported that only ≈50% of the Thy-1 antigen on thymoma cells and thymocytes was digested by phospholipase C. This result could be explained by extracellular matrix components shielding the remaining sites from enzymatic attack or by a portion of the protein population not being lipid-linked (29).

The validity of the photobleaching techniques has been questioned because many of the membrane protein diffusion coefficients have been smaller than expected (31). Photodamage artefacts during the photobleaching step or the size of the antibody ligand required to label the membrane protein have been postulated to produce these retarding effects. Rapid lateral diffusion might be expected for a glycoprotein, such as Thy-1, which is presumed to be anchored to the membrane via the glycolipid tail. It is reassuring that such rapid diffusion was measured by FRAP in this study, suggesting that, at least for the mobile fraction of Thy-1, the putative photodamage and labeling ligand size effects are not appreciable. In fact, recent FRAP measurements have shown that, under certain conditions, the $Na^+$ channel (32) and a fraction of the murine H-2 antigens (33) exhibit rapid lateral diffusion. Also, certain protein antigens on the surface of guinea pig sperm (34) and ram sperm (35) have diffusion coefficients in the range $10^{-9}$ to $10^{-8}$ cm$^2$/sec. All of these results give credence to the view that the range of lateral diffusion coefficients measured by FRAP for various plasma membrane proteins are not artefactual but have a biological origin and are worthy of serious interpretation.

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