

Lateral Diffusion of Membrane-spanning and Glycosylphosphatidylinositol-linked Proteins: Toward Establishing Rules Governing the Lateral Mobility of Membrane Proteins

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Abstract. In the plasma membrane of animal cells, many membrane-spanning proteins exhibit lower lateral mobilities than glycosylphosphatidylinositol (GPI)-linked proteins. To determine if the GPI linkage was a major determinant of the high lateral mobility of these proteins, we measured the lateral diffusion of chimeric membrane proteins composed of normally transmembrane proteins that were converted to GPI-linked proteins, or GPI-linked proteins that were converted to membrane-spanning proteins. These studies indicate that GPI linkage contributes only marginally (approximately twofold) to the higher mobility of sev-

eral GPI-linked proteins. The major determinant of the high mobility of these proteins resides instead in the extracellular domain. We propose that lack of interaction of the extracellular domain of this protein class with other cell surface components allows diffusion that is constrained only by the diffusion of the membrane anchor. In contrast, cell surface interactions of the ectodomain of membrane-spanning proteins exemplified by the vesicular stomatitis virus G glycoprotein reduces their lateral diffusion coefficients by nearly 10-fold with respect to many GPI-linked proteins.

It has been known for some time that membrane proteins reconstituted into fluid artificial bilayer membranes exhibit large lateral diffusion coefficients (D)¹ ($D > 10^{-8}$ cm²/s) (for review see Vaz et al., 1984), which can be reduced by approximately a factor of four or more by increasing the protein concentration (Peters and Cherry, 1982; Tank et al., 1982b; Scalettar and Abney, 1991). Studies on biological membranes confirmed this factor and roughly characterized the effect of crowding on lateral diffusion (Chazotte and Hackenbrock, 1988). However, many proteins in the plasma membrane exhibit diffusion coefficients considerably lower than this ($D \cong 10^{-10}$ cm²/s) (Peters, 1981; Jacobson et al., 1987). The implication is that structures peripheral to the plasma membrane, membrane associated cytoskeleton on the interior, and extracellular matrix (ECM) on the exterior, are somehow involved in restraining lateral mobility of membrane proteins beyond what is expected from protein concentration effects.

An approach to understanding the interactions limiting lateral mobility is based on expressing genetically engineered

mutant proteins which have defined structural alterations in one or more of their domains. Initial attempts focused on altering the cytoplasmic domains of various membrane proteins and were based on the erythrocyte Band 3 paradigm. Disruption (Golan and Veatch, 1980) or depletion (Sheetz et al., 1980) of the red cell cytoskeleton conferred a 40-fold increase in the lateral mobility of Band 3 indicating direct involvement of cytoplasmic domain interactions with the cytoskeleton in reducing Band 3 lateral mobility. However, in the case of the major histo-compatibility (MHC) antigens (Edidin and Zuniga, 1984), the EGF receptor (Livneh et al., 1986), and vesicular stomatitis virus G (VSV G) glycoprotein (Scullion et al., 1987), a number of drastic alterations in the cytoplasmic domains did not have a major effect on the lateral mobility of these proteins. For example, wild type VSV G exhibited similar lateral mobility to both TMR-Stop and G-IBV, having cytoplasmic domains of one and 128 residues, respectively (Scullion et al., 1987). These results suggest that different rules govern the lateral mobility of such membrane proteins. We therefore extended our efforts to the transmembrane and ectodomains of VSV G. Our earlier studies indicated that shortening the transmembrane domain and altering the number of glycosylation sites on VSV G had little effect on its lateral mobility (Scullion et al., 1987).

In this paper we have turned our attention to chimeras of membrane-spanning and glycosyl-phosphatidylinositol

1. *Abbreviations used in this paper:* D, diffusion coefficient; ECM, extracellular matrix; FRAP, fluorescence recovery after photobleaching; GPI, glycosyl-phosphatidylinositol; mf, mobile fraction; MHC, major histo-compatibility antigens; PLAP, human placental alkaline phosphatase; VSV G, vesicular stomatitis virus G glycoprotein.

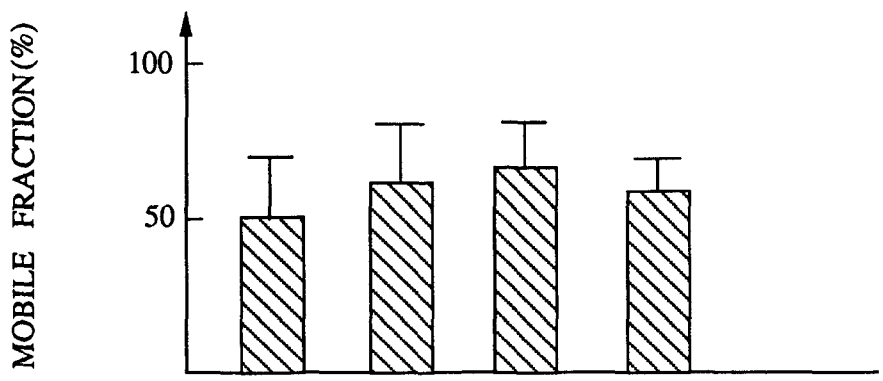
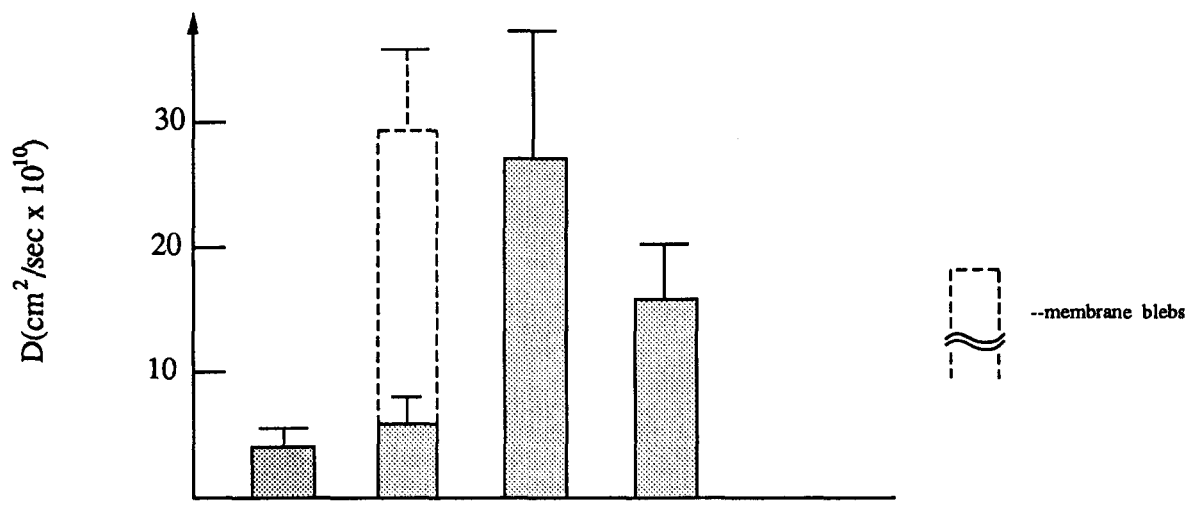
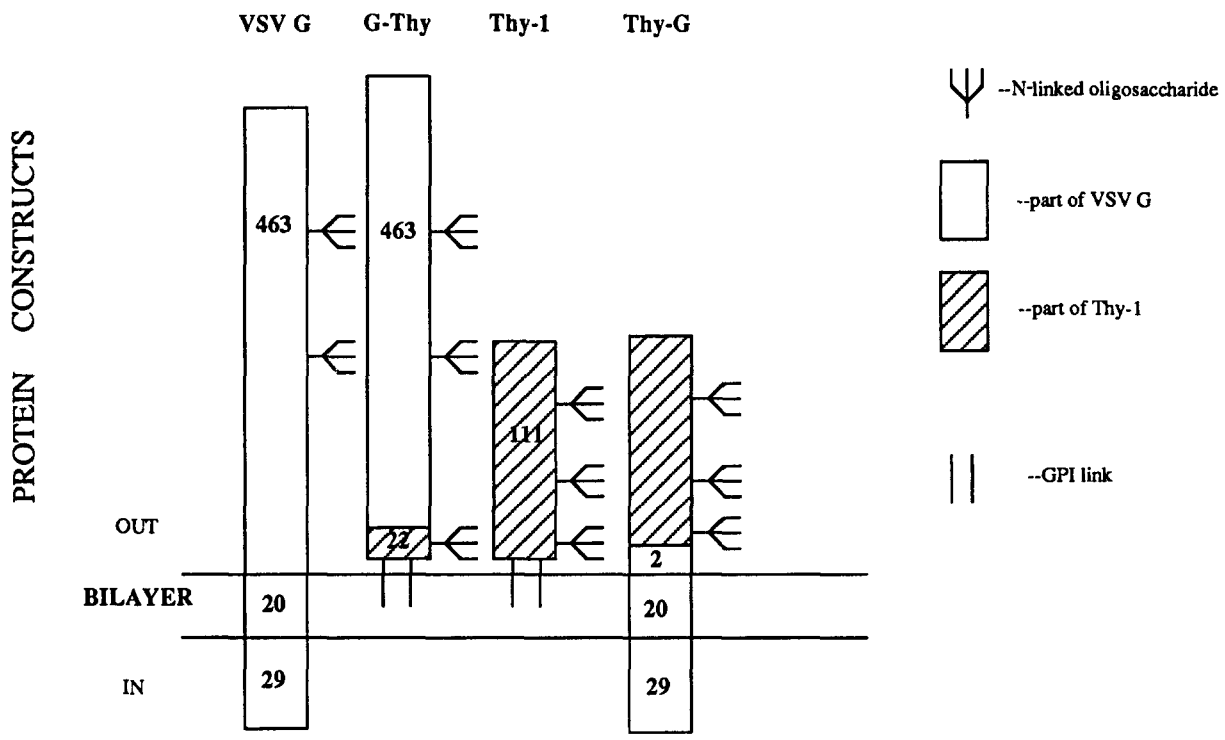


Table I. Lateral Mobility of Transmembrane Proteins*

Proteins	VSV G	G-hcG ₆ (complex poly-saccharides)	G-hcG ₆ (core poly-saccharides)	Thy-G	PLAP-G	Ly6E-D ^b
D(cm ² /s × 10 ¹⁰)	3.8 ± 1.8	3.2 ± 0.9	3.5 ± 1.1	16 ± 4	12 ± 4	17 ± 3
Mobile fraction (percent)	50 ± 20	62 ± 17	59 ± 22	58 ± 11	67 ± 15	60 ± 14
n	86	23	39	20	73	31

* Mean ± SD for D and mobile fraction.

Table II. Lateral Mobility of GPI-linked Proteins*

Proteins	Thy-1	G-Thy	G-Thy (bleb)	PLAP	Ly6E
D(cm ² /s × 10 ¹⁰)	27 ± 10	5.9 ± 2.1	29 ± 7	24 ± 6	28 ± 5
Mobile fraction (percent)	67 ± 14	62 ± 19	68 ± 22	70 ± 16	67 ± 14
n	32	46	5	44	25

* Mean ± SD for D and mobile fraction.

(GPI)-linked proteins and further ectodomain modifications to determine which protein domain plays a dominant role in determining lateral mobility. The fact that most transmembrane proteins exhibit lower lateral mobility than many GPI-linked proteins (Ishihara et al., 1987; Noda et al., 1987) was used as a rationale for producing several chimeras of the two groups of proteins. The transmembrane proteins used in these constructs were VSV G and the MHC class I D^b protein. The GPI-linked proteins used were Thy-1, a member of the Ig superfamily (Williams and Gagnon, 1982), human placental alkaline phosphatase (PLAP), a major cell surface enzyme (Knoll et al., 1988), and murine surface antigen Ly6E, a membrane protein involved in T cell activation (Rock et al., 1989).

Materials and Methods

Cells

COS-1 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in DME-H medium supplemented with 10% heat inactivated FCS and penicillin (100 U/ml) and streptomycin (100 µg/ml).

Vectors and Transfection

All the wild type and chimeric protein constructs were incorporated into SV-40 based vectors for expression in COS-1 cells as previously described (Crise et al., 1989; Berger et al., 1989; Su et al., 1991). Transfections were done using the DEAE-dextran method (Scullion et al., 1987). Cells plated on 12 × 12-mm coverslip in 35-mm dishes were incubated at 37°C by inverting the coverslip on a 25-µl droplet of PBS with Ca²⁺, Mg²⁺ (PBS+) containing 10 µg/ml of the appropriate plasmid, and DEAE-dextran (2 × 10⁶ mol wt) at a concentration of 500 µg/ml. After 30 min, 300 µl of complete medium containing 100 µM chloroquine was then added underneath the coverslip and the dishes returned to the 37°C incubator. After 3 h, the coverslips were rinsed with complete medium and placed in a new dish containing 2 ml of fresh medium. FRAP measurements were done 40 to 50 h after transfection.

Antibody Labels for FRAP Studies

Two anti-G mAbs were used, 8G5 and IE9; both were generous gifts of Dr. L. Lefrancois. The two anti-Thy-1 mAbs used, MRC Ox-7 and T24/31.7 were generous gifts of Drs. A. F. Williams and Ian Trowbridge, respectively. Affinity purified rabbit polyclonal anti-PLAP was obtained from Accurate Chemicals (Westbury, NY). mAb, SK70.94, was used against the Ly6E antigen.

All cells were labeled with rhodamine-conjugated Fab fragments of IgG mAbs (except for placental alkaline phosphatase (PLAP), which was labeled with Fab fragments from polyclonal IgG antibodies). The Fabs were prepared by papain digestion followed by protein-A column chromatography to remove intact IgG. The Fabs were free from IgG as assayed by SDS-PAGE and silver staining. Rhodamine conjugations were done as previously described (Scullion et al., 1987).

Production of Membrane Blebs

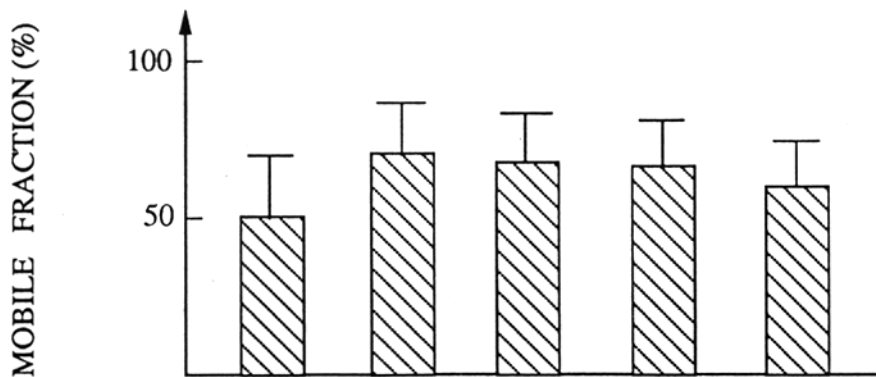
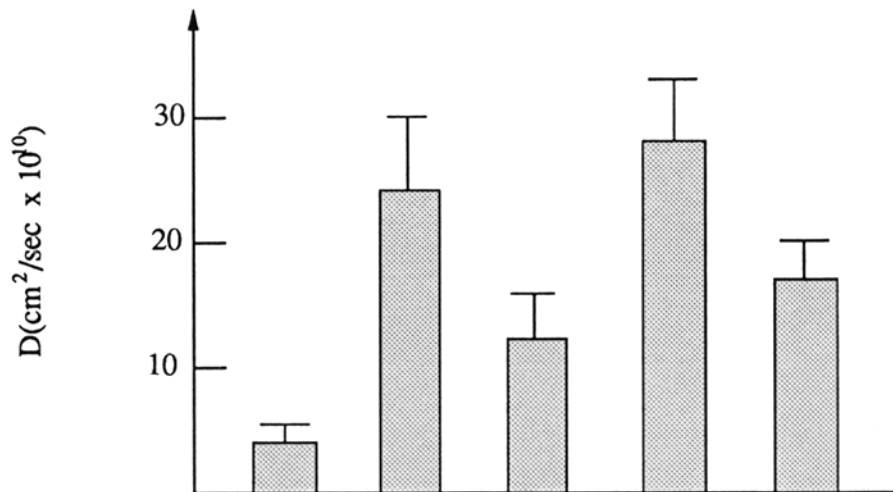
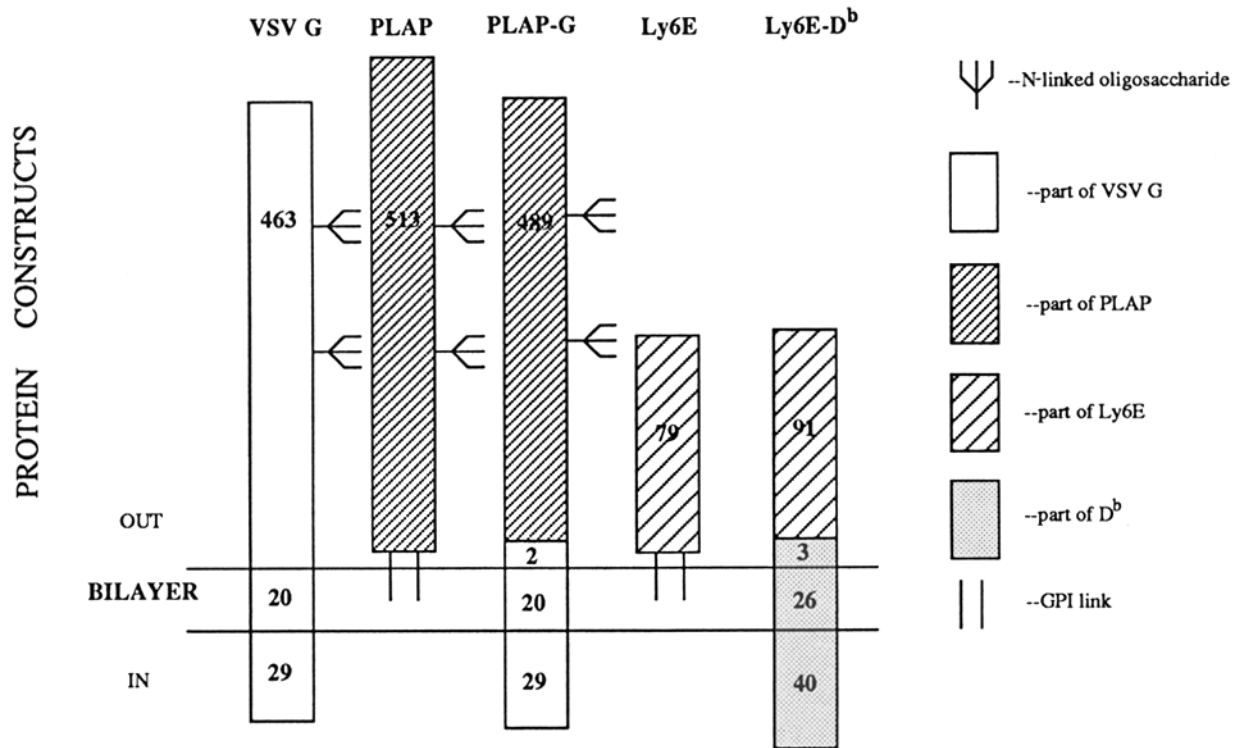
Cells were blebbed by brief exposure (5–20 s) to 2 M NaCl–NaOH (pH 10.0) and then labeled with rhodamine-conjugated Fab fragments.

FRAP Measurements

A Leitz 40× oil objective lens was used to focus the laser beam to a diameter of about 2.0 µm. The 514-nm line of a Spectra-Physics 164-07 argon 107 laser (Mountain View, CA) was used. Spots on the cell were bleached for 20 to 80 ms using a laser power of about 50 mW. The recovery phase was interrogated with beam powers of about 50 µW. After labeling the cells with rhodamine conjugated Fab fragments of the appropriate antibody, FRAP measurements were performed using continuous (Ishihara et al., 1987) or periodic (Scullion et al., 1987) monitoring of recovery. A single curve was recorded per transfected cell and each coverslip was used for less than one hour after antibody labeling.

Because an upward drift of the D values for wild type VSV G occurred over the course of 5 yr, we analyzed sources of systematic error in the physical measurement (Petersen et al., 1986). A major uncertainty lies in the determination of the laser beam radius, w_s , which we conservatively estimate at ±20%. This leads to ±40% uncertainty in the absolute value for the diffusion coefficient. In this regard, focusing the FRAP laser beam on the cell surface was more reproducible in the present study in that it was accomplished by imaging the beam using an image-intensified video camera instead of directly viewing the image of the beam through the eyepieces. Slight errors in focusing produce slightly different beam sizes intercepting the cell

Figure 1. Lateral mobility of chimeric constructs of VSV G and Thy-1. (Top) Schematic of protein constructs with the number of amino acids in the various domains and the number of glycosylation sites indicated. (Middle) Diffusion coefficient (D) of each protein construct. (Bottom) Mobile fraction of each protein construct.



surface, thus resulting in different recovery half times; different calculated diffusion coefficients arise because a single beam radius is assumed in the calculation. We estimate other errors due to beam alignment, curve fitting, etc., to be on the order of 10%. On this basis, it is likely that diffusion coefficients can be measured to an accuracy of $\pm 50\%$ and a precision, on uniform samples, approaching $\pm 10\%$ (Derzko and Jacobson, 1980) without elaborate precautions.

Cell populations can be heterogeneous and a wide range of D's are measured (Eddin and Wei, 1982; Jacobson et al., 1984). In this study we have found that uncertainty arises from data selection especially with regard to the assignment of the final fluorescence value, F^∞ . Because of cell movement the values of F^∞ at long times after bleach vary and even exceed 100% recovery. In the earlier work (Scullion et al., 1987), we tended to record recoveries for longer times, whereas for the present study we have truncated the recoveries when we consider excessive variability to have occurred. Effectively, this means we restrict our analysis to times equivalent to about 10 recovery half times. The new experimental and analysis modifications constitute a refinement of our techniques and therefore produce more accurate and precise data. The result is that mobile fraction (mf) are lower and recovery half times shorter (higher D's) than previously reported. We estimate from recalculating our previous data (Scullion et al., 1987) that this produces about a twofold increase in D for wild type VSV G ($8.5 \times 10^{-11} \text{ cm}^2/\text{s}$ to $1.65 \times 10^{-10} \text{ cm}^2/\text{s}$) and a 13% decrease in mf (75% to 62%). None of the biological conclusions from the earlier study are changed.

Results

Lateral Mobility of Membrane-spanning and GPI-linked Proteins

In Fig. 1, the lateral mobility data in terms of D (*middle*) and mobile fraction (*bottom*) for wild type VSV G, Thy-1, G-Thy, and Thy-G are compared. These proteins are schematically depicted in the top panel of Fig. 1 and numerical data is given in Table I and II. G-Thy, a GPI-linked glycoprotein, has the entire VSV G protein ectodomain and about 22 amino acid COOH terminus of the mature Thy-1 protein (Crise et al., 1989). Thy-G is a chimeric construct which has the coding sequence of the Thy-1 ectodomain fused with that of the VSV G transmembrane and cytoplasmic domains.

The lateral mobility of VSV G, expressed in transfected cells, is low. Thy-1 expressed in transfected cells exhibits high lateral mobility consistent with earlier studies (Ishihara et al., 1987). However, the lateral mobility of G-Thy is only slightly increased compared to that for VSV G; in fact, its lateral mobility is well below that characteristic of many GPI-linked proteins. This result indicated that the VSV G protein ectodomain is the prime determinant of the diffusion behavior of G-Thy. The mobile fraction of G-Thy is not significantly different from that of VSV G.

It has been shown that proteins in plasma membrane blebs exhibit higher lateral mobility and this has been interpreted as a result of the disruption of interactions between membrane proteins and submembranous cytoskeletal components in the blebbed regions (Tank et al., 1982a). As a control for our ability to measure fast diffusion, we measured the lateral mobility of G-Thy within blebbed regions of the COS-1 surface. We found that the lateral mobility was considerably higher and in fact approached what is considered the "diffusion limit" for integral proteins in the plasma membrane. (Fig. 1 and Table II). This result also points to the im-

portance of an intact peripheral structure in the restricted lateral diffusion of G-Thy.

On the other hand, the reverse construct produced by fusing the membrane spanning and cytoplasmic domains of VSV G to the ectodomain of Thy-1 (Thy-G) results in only a less than twofold reduction in D compared to that for Thy-1. The lack of a large, order of magnitude, effect is consistent with the small effects of major cytoplasmic domain alterations on the diffusion of VSV G (Scullion et al., 1987). Our measurement indicates that changing the mode of membrane anchorage from lipid to peptide reduces lateral diffusion by less than a factor of two, not unlike differences between peptide and lipid diffusion in artificial bilayer systems (Wu et al., 1978; Tank et al., 1982b).

A similar situation is shown for human PLAP and PLAP-G in Fig. 2 and Tables I and II. PLAP-G is a transmembrane form of PLAP (Berger et al., 1989), which has the entire sequence encoding mature PLAP fused with transmembrane and cytoplasmic domains of VSV G glycoprotein (Fig. 2, *top*). A partially homologous and endogeneous rat osteoblastic alkaline phosphatase exhibits a large lateral diffusion coefficient (Noda et al., 1987) and the data for PLAP expressed in transfected cells are consistent with this measurement. The PLAP-G construct, which retains enzymatic activity, exhibits lateral mobility which is among the highest measured for transmembrane plasma membrane proteins but which is slightly lower than that of wild type GPI-linked forms. In fact, the decrement in lateral mobility between PLAP and PLAP-G parallels that seen when Thy-1 and Thy-G are compared (Fig. 1).

Another pair of GPI linked and membrane spanning antigens gave similar lateral mobility results. Ly6E-D^b (Su et al., 1991; Su and Bothwell, 1989) is a transmembrane form of Ly6E, having the entire sequence of the Ly6E ectodomain fused to the transmembrane and cytoplasmic domains of the MHC class I antigen D^b. Lateral mobility data are given in Fig. 2 and Tables I and II. In this case as well, fusing membrane spanning and cytoplasmic domains from the MHC protein reduced D for the chimera by less than a factor of two as compared to the native GPI-linked antigen.

In contrast to the results with Thy-G and PLAP-G, one other switch in the ectodomain of VSV G could be made without significantly altering its lateral mobility from that of the wild type. Substitution of the normal VSV G ectodomain with the 12 kD α subunit of human chorionic gonadotropin (GhcGa; Guan et al., 1988), a normally secreted product, had little effect on this chimera's lateral mobility as compared to wild type VSV G (Table I). This chimeric mutant has a long polylactosaminoglycan chain added to its N-linked polysaccharide; however, inhibition of the polylactosaminoglycan assembly by 1-deoxymannojirimycin (Fukuda et al., 1988) did not alter its lateral mobility within the experimental uncertainty (Table I). Presumably, this is because this foreign ectodomain together with core glycosylation alone is sufficient to reduce lateral mobility.

In this study, we have referenced our D values to the value for wild type VSV G; it must be emphasized that these values

Figure 2. Lateral mobility of PLAP, PLAP-G, Ly6E, and Ly6E-D^b. (*Top*) Schematic of protein constructs with the number of amino acids in various domains and the sites of glycosylation indicated. (*Middle*) Diffusion coefficient of each protein construct. (*Bottom*) Mobile fraction of each protein construct.

Table III. Effects of Antibody Label on VSV G Lateral Mobility Measurement*

Antibody preparation	8G5		IE9		Fab/IgG [‡] mixture
	Fab	IgG	Fab	IgG	
D(cm ² /s × 10 ¹⁰)	3.8 ± 1.8	2.8 ± 1.3	4.2 ± 2.1	1.7 ± 0.6	4.3 ± 2.1
Mobile fraction (percent)	50 ± 20	52 ± 23	63 ± 20	27 ± 16	51 ± 23
n	86	20	43	37	22

* Mean ± SD for D and mobile fraction.

‡ 15% of IE9 IgG + 85% of IE9 Fab.

remained consistent over the course of the studies. However, baseline D values have drifted upward over the course of five years since our studies began from 0.85×10^{-10} cm²/s (Scullion et al., 1987) to 3.6×10^{-10} cm²/s (present study). Such drift could be caused by systematic error in the physical measurement and analysis, variability in the labeling antibody preparation, and biological changes in the COS cell expression system. The change in D values observed resulted mainly from improved analysis of the data (see Materials and Methods). Variability in the antibody preparation and unspecified biological drift in the COS cell expression system appear to be minor, if not negligible factors (see control studies below). Our analysis indicates that the effect of the mutations in the previous paper was, if anything, smaller than stated, with the largest deviation of mutant and control being approximately twofold rather than threefold. Thus, the biological conclusions of our earlier study (Scullion et al., 1987) remain unaltered and are, in fact, strengthened: major structural variations in the three domains of VSV G can be made without increasing its lateral mobility appreciably to values anywhere near approaching the "diffusion limit" found on membrane blebs or reconstituted lipid bilayers (Jacobson et al., 1987).

Control Studies on the Antibody Labels and COS Cell Expression System

It is of interest for photobleaching studies in general that our FRAP results were comparable for two different anti-G mAbs (8G5 and IE9 in Table III). Similar results were also obtained for two different mAb directed toward Thy-1 on AKR mouse lymphoma cells (data not shown). These results suggest that the site where the mAb decorates these antigens does not produce a large effect on the measured D.

In addition, we checked the influence of possible IgG contamination of the mAb preparations. Since VSV G is expressed as a trimer (Doms et al., 1987), a small amount of IgG or aggregated IgG contamination could cause cross-linking leading to a reduced D and/or reduced mobile fraction. Indeed, these effects were observed when the Fab results are compared to the results using intact IgG for two different mAb (compare columns 1, 2 for 8G5 and 3, 4 for IE9). The Fab preparations were centrifuged at 175,000 g for 30 min before use to guard against aggregation producing multivalent complexes of the Fab fragments; the FRAP values obtained were not significantly different after centrifugation of the Fab fragments, however. Next, a small amount of labeled IgG was added to the Fab preparation. The average values for D and mf did not change much from when pure Fab was used. However, the mean mf decreased and two popu-

lations of mobile fractions appeared, one at ~25% and the other at 65%. The fact that such multiple mf populations did not occur in the experimental data sets suggests that functionally significant IgG contamination of the Fab preparation was negligible.

To check for the stability of the expression system in regard to lateral mobility, we thawed COS cells from 1986 and 1989 batches, transfected the cells, and probed the expressed wild type G with rhodamine-labeled Fab fragments of IE9 mAb. The D's and mobile fractions were identical within experimental error (1986 COS cells: D = $4.3 [\pm 1.8] \times 10^{-10}$ cm²/s; mf = 69 ± 17 [n = 24]; 1989 COS cells: D = $4.2 (\pm 2.1) \times 10^{-10}$ cm²/s; mf = 63 ± 20 [n = 43]). This result indicated that the COS cells were stable with regard to the lateral diffusion of the VSV G protein.

Discussion

On the basis of this work, we suggest that two classes of mobile proteins exist in the plasma membrane: proteins which move at or close to the diffusion limit and those which move appreciably slower. Many GPI-linked proteins fall in the first class although there are notable exceptions (see below). The lateral diffusion coefficients of these proteins would be limited only by those factors which determine the mobility of the membrane anchor within the plane of the bilayer, including lipid composition and phase structure, and protein concentrations. These GPI-linked proteins appear to have "slippery" ectodomains which do not interact significantly with cell surface structures. In contrast, the ectodomain is implicated directly in restricting the lateral mobility of certain single spanning membrane glycoproteins, such as VSV G.

An anonymous referee has pointed out that the frictional coefficients of the individual domains of the protein would add linearly which should allow the contributions of each domain to the total frictional coefficient (f_{total}) to be dissected. (In fact, this statement is strictly true only when there is no hydrodynamic interaction between the various domains; Cantor and Schimmel, 1980.) Thus, f_{total} can be written as:

$$f_{\text{total}} = f_{\text{ecto}} + f_{\text{anchor}}, \quad (\text{a})$$

where f_{ecto} and f_{anchor} are the frictional coefficients of ectodomain and the membrane anchoring domains, respectively. In the case of VSV G, the transmembrane anchor is considered to be both the membrane-spanning peptide and the small cytoplasmic domain, whereas in the case of Thy-1, the anchor is the GPI tail. The total frictional coefficient can be calculated from the measured D using the Einstein relation:

Table IV. Gross Structural Properties of Glycoprotein Ectodomains

Glycoprotein	Membrane anchorage	D* (cm ² /s × 10 ¹⁰)	Number of amino acid residues	Oligomeric state	Number of N-linked glycosylation sites
VSV G	TM	3.8	463	Trimer (Doms et al., 1987)	2
G-hcG _α	TM	3.2	92	Monomer [‡]	2
Thy-1	GPI	27	111	Monomer [§]	3
PLAP	GPI	24	513	Dimer (Millan, 1986)	2
Ly6E	GPI	28	79	Monomer (Palfree et al., 1986)	0

* All FRAP measurements made using Rh-Fab fragments of appropriate antibodies.

[‡] G-hcG_α from unpublished experiments.

[§] Chemical crosslinking experiments of S. Easterbrook-Smith did not indicate crosslinked forms of Thy-1 (A. Williams, personal communication).

[†] TM, membrane-spanning peptide; GPI, lipid linkage.

$$f_{\text{total}} = kT/D, \quad (\text{b})$$

where k is the Boltzmann constant, T is the absolute temperature, and D is the measured diffusion coefficient.

From our experimental data, four relationships are obtained:

$$f_G = f_{G\text{-ecto}} + f_{G\text{-anchor}} = (2.6 \pm 1.3) \times 10^{-1} kT, \quad (\text{c})$$

$$f_{\text{Thy}G} = f_{\text{Thy-ecto}} + f_{G\text{-anchor}} = (6.3 \pm 1.6) \times 10^{-2} kT, \quad (\text{d})$$

$$f_{\text{Thy}} = f_{\text{Thy-ecto}} + f_{\text{Thy-anchor}} = (3.7 \pm 1.4) \times 10^{-2} kT, \quad (\text{e})$$

$$f_{G\text{Thy}} = f_{G\text{-ecto}} + f_{\text{Thy-anchor}} = (1.7 \pm 0.6) \times 10^{-1} kT, \quad (\text{f})$$

where the frictional coefficients are in units of erg-s/cm². Since these four equations are not independent, there is no unique solution. However, it is reasonable to assume that the GPI anchor of Thy-1 will have a lipidlike D value of 30×10^{-10} cm²/s, so that:

$$f_{\text{Thy-anchor}} = kT/30 \cong 3.3 \times 10^{-2} kT$$

Substituting $f_{\text{Thy-anchor}}$ into (e) yields:

$$f_{\text{Thy-ecto}} = kT/27 - kT/30 \cong (3.7 \pm 1.4) \times 10^{-3} kT.$$

Similar substitutions in (f), (d), and (c) yield:

$$f_{G\text{-ecto}} = kT/5.9 - kT/30 \cong (1.4 \pm 0.6) \times 10^{-1} kT,$$

$$f_{G\text{-anchor}} = kT/16 - f_{\text{Thy-ecto}} \cong (5.9 \pm 2.2) \times 10^{-2} kT$$

or $f_{G\text{-anchor}} = kT/3.8 - f_{G\text{-ecto}} \cong (1.2 \pm 1.4) \times 10^{-1} kT,$

This analysis yields the following values for the individual

domain frictional coefficients relative to that for the Thy anchor:

$f/f_{\text{Thy-anchor}}$			
Thy-anchor	Thy-ecto	G-ecto	G-anchor
1.0	0.11	4.1	1.8, 3.7

Values obtained for G-anchor depend on whether equation (d) or (c) is used.

Thus, some quantification of the impediments to diffusion provided by the interactions of individual domains with their local environments can be accomplished. For example, the ectodomain of the Thy-1 has a frictional coefficient some 40-fold smaller than that for the VSV G ectodomain. In fact, it offers less impediment to diffusion than the Thy-1 GPI anchor. In addition, the peptide anchor together with its small cytoplasmic domain offers a two- to fourfold larger impediment to diffusion than the GPI anchor.

At a gross structural level there is little to suggest what properties of ectodomains permit them to severely restrict lateral diffusion or to be relatively "slippery." As shown in Table IV, the lateral diffusion coefficient does not correlate in an obvious way with ectodomain size, oligomeric state, or degree of glycosylation. Extracellular matrix could interact preferentially with membrane-spanning proteins; however, much of the extracellular matrix structure on the cell surface is coarsely distributed in prominent fibrillar structures (Hayman et al., 1982; Hedman et al., 1982; Bornstein

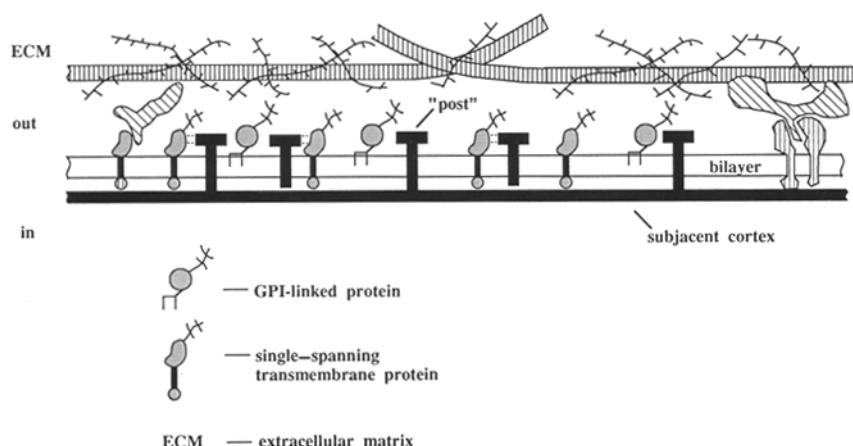


Figure 3. Transient interaction model to explain low diffusion coefficient of many membrane-spanning glycoproteins.

and Ash, 1977) as depicted schematically in Fig. 3. Thus, the extracellular matrix could be envisioned as contacting the membrane via receptors in discrete regions but not providing a sufficiently high and uniform surface density of slowly diffusing or immobile binding sites required to retard mobility over the entire cell surface (see Jacobson et al., 1984). Moreover, immunostaining indicates that COS cells, while expressing small amounts of cell surface fibronectin and collagen in a punctate, nonfibrillar array, are notably lacking in laminin, and vitronectin (data not shown). We therefore favor the following interpretation involving the membrane associated cytoskeleton.

The lower mobility of the majority of membrane-spanning proteins must be because of additional interactions with intra- or extramembrane structures. We base an explanation of this phenomenon on several postulates. First, transient, rapid exchange interactions of the diffusing protein with slowly diffusing or immobilized structures serve to reduce the diffusion coefficient (see Jacobson et al., 1984 and references therein) because relatively low affinity interactions between proteins can be significant in membranes. Grasberger et al. (1986) have pointed out that high protein concentration, excluded volume effects, and the lack of complete rotational freedom (which makes association less costly entropically), all combine to favor membrane protein association even if relatively weak binding constants prevail.

Second, the mobility of the diffusing protein is retarded by interactions with a system of "post" proteins which are "anchored" to the cortical cytoskeleton (Fig. 3). Such proteins may bear some similarity to the "agorins" which are proposed to form a plasma membrane skeleton in lymphoid cells (Apgar and Mesher, 1986) or the Na⁺-K⁺ ATPase in polarized epithelium which is localized to the basolateral domain in complexes with ankyrin and fodrin (Nelson and Hamerton, 1989). These post proteins are hypothesized to be laterally immobile or nearly so. Such post proteins could fit the Band 3 paradigm in which a relatively long, flexible cytoplasmic domain is either anchored to the subjacent cytoskeleton or becomes entrapped in the matrix created by the membrane associated cytoskeleton. This entrapment impedes long range lateral diffusion (Koppel et al., 1981; Saxton, 1990; Tsuji and Ohnishi, 1986) while permitting rotational diffusion of a fraction of the proteins (Nigg and Cherry, 1980).

In many cases the critical interactions may occur between the ectodomains of the post proteins and the diffusing proteins. For single spanning proteins having small cytoplasmic domains, the ectodomain interactions may predominate because of steric reasons (Fig. 3). In the case of the class I major histocompatibility antigens, the number of glycosylation sites in the ectodomain appears to be an important contributor to mobility retarding interactions (Wier and Edidin, 1988). However this factor does not appear to be important in determining the lateral mobility of VSV G (Scullion et al., 1987).

Postulating that interactions between the diffusant and the posts are in rapid exchange means that, with reference to the mobile fraction, the observed diffusion coefficient D is given by: $D = f_f D_f + f_b D_b \cong f_f D_f$, where D_f and D_b and f_f and f_b represent the D 's and the fractions of freely diffusing protein and protein bound to the posts, respectively (see Jacobson et al., 1984). In this model, lateral diffusion of the glycopro-

teins is limited by short dwell-time interactions with the putative posts between which the protein takes fast diffusive steps through the bilayer; the mobility exhibited during these fast steps is governed by membrane "fluidity." Since the fast steps are characterized by a diffusion coefficient of $D_f \geq 3 \times 10^{-9}$ cm²/s (see above) and the posts are slowly diffusing or immobile ($D_b \leq 10^{-11}$ cm²/s), the approximation in the equation above is justified. The observed D is thus directly proportional to the diffusion coefficient that the protein exhibits between "visits" to the posts. This offers an explanation for why G-Thy has a slightly larger diffusion coefficient than VSV G: in general, based on studies in model membranes (Wu et al., 1978; Tank et al., 1982b), lipid anchors would be expected to confer slightly greater mobility on membrane proteins than do peptide anchors. This difference is apparently largely because of the fact that peptide anchors span the membrane, whereas GPI anchors do not: bilayer spanning lipid derivatives have diffusion coefficients $\sim 2/3$ as large as similar lipids moving in one monolayer only (Vaz et al., 1985).

Not all GPI-linked proteins exhibit large lateral diffusion coefficients. PH-20, a GPI-linked surface antigen exhibits highly restricted diffusion on testicular sperm before epididymal maturation (Phelps et al., 1988) and VSG, the variant surface glycoprotein of trypanosoma diffuses slowly both on the parasite surface and when inserted into cultured mammalian cells (Bülow et al., 1988). These GPI-linked glycoproteins appear to have their lateral mobility restricted by ectodomain interactions, perhaps with putative post proteins. Alternatively, nuclear magnetic resonance studies and molecular calculations of VSG structure indicate that its glycan moiety adopts an extended configuration positioned between the VSG protein and the bilayer (Homans et al., 1989). Thus, it is possible that interactions between the glycan moiety and the bilayer surface restrict diffusion of this GPI-linked glycoprotein.

In contrast to single-spanning transmembrane proteins, some multiple spanning and multi-subunit membrane proteins do exhibit a dependency of their diffusion coefficient on the cytoplasmic domain(s). Trypsin cleavage of the Band 3 cytoplasmic domain resulted in nearly an order of magnitude increase in its D (Tsuji and Ohnishi, 1986). Cytoplasmic domain truncation of either or both subunits of the heterodimeric class II MHC molecules does increase their diffusion coefficients (Wade et al., 1989). As mentioned above, proteins of this type could serve as the putative "posts."

The lateral regionalization of membrane components is of great functional importance. All membrane proteins, whether lipid linked or transmembrane, exhibit an immobile fraction which is presumably related to microheterogeneity in the plane of the membrane (Wolf, 1987; Yechiel and Edidin, 1987; Sheetz et al., 1989; Edidin and Stroynowski, 1991; deBrabander et al., 1991, for example). Furthermore, Edidin and Stroynowski (1991) have found that some GPI-linked proteins exhibit a lower apparent diffusion coefficient at very small beam radii but an immobile fraction independent of beam radius, whereas transmembrane proteins have both reduced D 's and increased mobile fractions at small beam sizes. Edidin and co-workers (1987, 1991) interpret their data as indicating transmembrane protein confinement to micron-sized protein rich domain structures in the plane of the plasma membrane; however, the GPI-linked proteins

would not be restricted to these domains. Our studies were performed at beam radii ($\sim 1.1 \mu\text{m}$) large enough so that beam size effects would be minimal. Therefore, our data would most likely correspond to an interrogation of regions large enough to encompass both the continuum between domains and several of the putative protein rich domains.

A major issue regarding the use of protein expression in COS cells is whether expression of foreign proteins will be subject to interactions similar to what native membrane proteins experience (Wade et al., 1989). In this regard, it is encouraging that the diffusion coefficient for the GPI-linked protein, Thy-1, when expressed in COS cells is similar to values measured for the endogenously expressed protein (Ishihara et al., 1987). This suggests that general principles of mobility regulation are preserved in the COS cell.

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