Differences between the Lateral Organization of Conventional and Inositol Phospholipid-anchored Membrane Proteins. 
A Further Definition of Micrometer Scale Membrane Domains

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Abstract. Plasma membranes of many cells appear to be divided into domains, areas whose composition and function differ from the average for an entire membrane. We have previously used fluorescence photobleaching and recovery to demonstrate one type of membrane domain, with dimensions of micrometers (Yechiel, E., and M. Edidin. 1987. J. Cell Biol. 105: 755-760). The presence of membrane domains is inferred from the dependence of the apparent mobile fraction of labeled molecules on the size of the membrane area probed. We now find that by this definition classical class I MHC molecules, H-2Dk, are concentrated in domains in the membranes of K78-2 hepatoma cells, while the nonclassical class I-related molecules, Qa-2, are free to pass the boundaries of these domains. The two proteins are highly homologous but differ in their mode of anchorage to the membrane lipid bilayer. H-2Dk is anchored by a transmembrane peptide, while Qa-2 is anchored by a glycosylphosphatidylinositol (GPI) anchor. A mutant class I protein with its external portion derived from Qa-2 but with transmembrane and cytoplasmic sequences from a classical class I molecule shows a dependence of its mobile fraction on the area of membrane probed, while a mutant whose external portions are a mixture of classical and nonclassical class I sequences, GPI-linked to the bilayer, does not show this dependence and hence by our definition is not restricted to membrane domains.

CELL surface membranes are differentiated into patches or domains whose composition, physical properties, and function differ from the average for an entire cell surface. Some membrane domains, for example the basal/lateral or apical surfaces of morphologically polarized epithelial cells (Rodriguez-Boulan and Nelson, 1989), are easily seen by light microscopy and even domains as small as 50-100-nm clusters of glycolipids are seen in electron micrographs of erythrocyte membranes (Thompson and Tillack, 1985). Other membrane domains of various sizes are also detected in cell surface membranes (reviewed in Edidin, 1990a) though they usually cannot be imaged in micrographs. Instead, their presence is inferred from the behavior of membrane probes that is not predicted by, or consistent with, the properties of a continuous, fluid, lipid bilayer. Such domains then are defined by the experiments that detect them, for example in terms of heterogeneities of fluorescence lifetimes (Klausner et al., 1980; Karnovsky et al., 1982) or of lateral diffusion coefficients (Wolf et al., 1981; Handrysie et al., 1982; Wolf et al., 1988).

We have defined one type of domain using fluorescence photobleaching and recovery (FPR) to measure the mobile fractions of a lipid probe and of labeled proteins in human fibroblast cell surface membranes (Yechiel and Edidin, 1987). In an FPR experiment, diffusion of a fluorescent label is measured in terms of the return of fluorescence to a small area of the surface that has been partly bleached by a focused laser beam. The time for recovery of fluorescence is expected to depend upon the area bleached, but the extent of recovery of fluorescence, which depends on the fraction of mobile molecules in the labeled population, is expected to be independent of the area. While this is true in synthetic bilayer membranes, we found that in human fibroblast plasma membranes the mobile fraction of a fluorescent lipid analogue, NBD-PC and of antibody-labeled proteins does depend on the area bleached, even though these areas, 1-25 μm² are significantly smaller than that of the cell surface, 2-300 μm² (Yechiel and Edidin, 1987). These observations together with a detailed analysis of diffusion coefficients were interpreted in terms of protein-rich domains, ~2 μm in diameter, interspersed with lipid-rich regions of membrane. Structures of about this size were in fact observed when large areas (100-200 μm²) of membrane brightly labeled with NBD-PC were repeatedly bleached and then observed with low-intensity laser light.

A better definition of the structure of the micrometer scale domains defined by FPR experiments could be gained by the study of a single protein with variant forms of anchoring to

1. Abbreviations used in this paper: FRP, fluorescence photobleaching and recovery; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex.
cytoplasmic tail. The GPI of Qa-2 is thought to be attached to the COOH-terminal residue of the alpha 3 domain or to a downstream amino acid encoded by exon 5 (Ullrich et al., 1990). Qa-2 is expressed on K78-2 transfected HEPA OVA cells. QQQD is expressed on K123-12 cells and QQQD is expressed on K123-13 cells. All of these cells express H-2Db.

the membrane bilayer. Class I major histocompatibility complex (MHC) molecules allow such a comparison since there are many variants and mutants of each of the molecule's domains, extracellular, transmembrane, and cytoplasmic. Here we compare the diffusibility of two class I MHC molecules which are highly homologous in their external domains: a classical class I molecule, H-2Db, that is anchored to the lipid bilayer by a transmembrane peptide, and a nonclassical class I-related molecule, Qa-2 (Stroynowski, 1990), that is glycosylphosphatidylinositol (GPI)-anchored to the bilayer (Stroynowski et al., 1987). These molecules show different behavior in FPR experiments when expressed in the same cell surface. The mobile fraction of H-2Db antigens depends upon the area of the membrane interrogated in an FPR experiment, while the mobile fraction of Qa-2 antigens is independent of this area. Using our operational definition of a membrane domain we conclude that Qa-2 antigens are able to cross domain boundaries in the time (minutes) of an FPR experiment. This suggests that transmembrane and cytoplasmic regions of proteins are important in keeping them organized in micrometer scale domains.

**Materials and Methods**

**Cells and Genes**

The recipient HEPA-OVA mouse hepatoma cells (Darlington et al., 1980) express endogenous H-2Db MHC molecules, but not H-2Kb molecules (Fig. 1). K78-2 are HEPA OVA cells transfected with a Qa-2 gene (Q7b). They express transmembrane-spanning H-2Db and glycosphospholipid-linked Qa-2 molecules (Fig. 1). K123-12 are HEPA-OVA transfected with and expressing the product of a hybrid class I gene specifying the alpha 1, alpha 2, and alpha 3 domains of Qa-2 and transmembrane and cytoplasmic domains identical to H-2Db (Fig. 1). A subpopulation of cells was selected by three rounds of flow cytometry; Epics Division, Coulter Corp., Hialeah, FL). The original population consisted of cells only about two times brighter than unlabeled controls. Sorting of the brightest and dimmest cell populations for the brightest 5-10% of labeled cells (Epics 752 flow cytometer; Epics Division, Coulter Corp., Hialeah, FL). Unreacted fluorescein was removed by passing the conjugates over Sephadex G-25.

Conjugates were ultracentrifuged for 1 h at 100,000 g (Airfuge, Beckman Instruments, Inc., Fullerton, CA) before use in FPR experiments (Bierer et al., 1987).

**Fluorescent Lipid Analogue**

NBD-PC ([1-palmitoyl-2-[6-[7-nitro-2-1-3-benoxiadiazol-4-y l](amino)crotyl] phosphatidylcholine] was obtained from Avanti Biochemical, Inc. (Birmingham, AL). It was diluted into medium from an ethanol stock solution to give a final concentration of 0.1 µg/ml dye and 1% ethanol. Cells were labeled in this solution for 30 min on ice. dim C16 (1,1-diheptadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate) was obtained from Molecular Probes, Inc. It was diluted into medium from a 100 µg/ml stock in ethanol to give a final concentration of 0.5-1.0 µg/ml dim.
The mobile fractions of NBD-PC in K78 hepatoma cell membranes when measured with laser spots of different radii. Data, shown as mean ±SEM, are pooled from three experiments. One experiment consists of one day's measurements that may be made on one or more cell samples.

**FPR**

Our spot FPR machine and software have been described elsewhere (Wolf and Edidin, 1981). The machine is based on a Leitz Ortholux microscope, coupled to a 3.5 W argon ion laser (Coherent Radiation Inc., Palo Alto, CA). An E G & G photon counter outputs pulses to a custom counter/timer board controlled by software. The measuring laser power density was typically 0.4–4 kW/cm²; the bleaching power density was 0.8–1 MW/cm².

Cells to be used for FPR were grown on 25-mm square coverslips in medium containing 10–30% of the supernatant from Con A-stimulated rat T cells. Exposure to this crude interferon-containing medium increased expression of QA-2 and H-2D<sup>b</sup> about two-fold after 24 h.

Regions for measurement were selected in bright field (phase contrast) and were chosen to be away from the nucleus. No selection was made in terms of fluorescence intensity which varied three- to fourfold in the cultures used. Only one measurement was made for each cell. Occasionally the region chosen was very bright, five to ten times greater than average. Fluorescence in these spots was always immobile and presumably was due to fluorescent debris stuck to the cells.

**Determination of the Radii of the Spots Produced by Different Objectives**

The accurate determination of laser spot size is critical to the interpretation of our experiments. We determined the spot sizes produced by different objectives by a variant of method three of Schneider and Webb (1981), scanning the intensity profile of an expanded image of the laser spot, but using a video camera instead of film. The expanded image was obtained by substituting a piece of plain glass for the dichroic mirror and filter so that the laser light could be reflected off of a first surface mirror and projected into the video camera. Scratches in the mirror allowed careful focusing onto its surface. The scans were recorded using a video line analyzer (model 321; Colorado Video, Boulder CO) whose output was recorded on a chart recorder. The tracings of intensity profiles were calibrated against scans of a stage micrometer divided into 2-μm rulings. The system's response, calibrated with neutral density filters, is linear down to 4% of the maximum (subsaturation) intensity used. Hence we can directly measure the e<sup>−2</sup> height above baseline and determine the beam diameter at this level. We have also used the approach of Schneider and Webb (1981), measuring the height of the peak with a 50% transmission neutral density filter in the light path and using this height to define the diameter of the unfiltered image. Results by the two methods agreed within 20% or less despite small inaccuracies in orienting the filters normal to the laser beam.

If we have accurately measured spot sizes, and there is no systematic error in measurement, then, while <i>t</i> should equal to that calculated <i>D</i> for diffusion of a protein in a homogeneous model system. We made a simple model by making a solution of 2% fluorescent IgG in water/98% anhydrous glycerol (wt/wt). The mixture was equilibrated for 4 d before use in FPR. Its viscosity at 23°C was 654 ± 40 centipoise. Values of <i>D</i> (averaged from 10 measurements) for different objectives were: 90× (0.357-μm-spot radius) 5 × 10<sup>−10</sup> cm² · s<sup>−1</sup>; 63× (0.45-μm-spot radius) 4 × 10<sup>−10</sup> cm² · s<sup>−1</sup>; 40× (0.75-μm-spot radius) 4 × 10<sup>−10</sup> cm² · s<sup>−1</sup>; 22× (1.3-μm-spot radius) 5 × 10<sup>−10</sup> cm². These <i>D</i> differ <20% from <i>D</i> calculated from the published <i>D</i> for IgG in water (4 × 10<sup>−7</sup> cm² · s<sup>−1</sup>), and the viscosity of our glycerol solution corrected for the measurement temperature, 20°C. Complete recovery of fluorescence was seen for all spot sizes (average mobile fraction = 1).

We found no dependence of the measured diffusion coefficient on the depth of bleaching in the range 25–75% for any objective except the 90×. The 90× objective gives higher <i>D</i> with increasing bleaching depths. This probably reflects the fact that our program for analysis of FPR curves extrapolates the observed recovery back to a time in the mid-point of the bleach. We have found, using 95% glycerol solutions that the 90× objective can reliably resolve <i>D</i> of 1–2 × 10<sup>−9</sup> cm² · s<sup>−1</sup>, but that larger <i>D</i> are not reliably determined with this objective. Instead, the recovery curves are truncated so that <i>D</i> appears to be ~10-fold smaller than it actually is.

**Results**

The experiment that we originally used to define domains in fibroblast plasma membranes (Yechiel and Edidin, 1987) shows that the similar domains exist in the plasma membranes of K78-2 hepatoma cells. Data from three experiments on the relationship between mobile fraction and beam radius for NBD-PC-labeled K78-2 cells and covering a range of three to 1.7 μm are summarized in Fig. 2. While there is a range of mobile fractions among cells examined at any one beam radius, it is clear that the apparent mobile fraction decreases as the size of the illuminated region of the cell surface increases. This is not an artifact of the microscope optics since it is not seen for NBD-PC incorporated into large, cell-sized, liposomes (Yechiel and Edidin, 1987), for diffusion of fluorescence protein in glycerol.
Figure 4. The mobile fractions of H-2D\(^\beta\) (a–c) or Qa-2 (d–f) molecules in membranes of individual K78 hepatoma cells when measured with laser spots of different radii. (a) H-2D\(^\beta\). Spot radius, 0.4 \(\mu\)m (63× objective). Average mobile fraction is 0.53 ± 0.13. (b) H-2D\(^\beta\). Spot radius, 0.6 \(\mu\)m (40× objective). Average mobile fraction is 0.39 ± 0.08. (c) H-2D\(^\beta\). Spot radius, 1.1 \(\mu\)m (22× objective). Average mobile fraction is 0.32 ± 0.12. (d) Qa-2. Spot radius, 0.3 \(\mu\)m (90× objective). Average mobile fraction is 0.55 ± 0.14. (e) Qa-2. Spot radius, 0.7 \(\mu\)m (40× objective). Average mobile fraction is 0.53 ± 0.15. (f) Qa-2. Spot radius, 1.1 \(\mu\)m (22× objective). Average mobile fraction is 0.54 ± 0.15.

Figure 5. The mobile fractions of Fi-Fab-labeled H-2D\(^\beta\) molecules in K78 hepatoma cell membranes when measured with laser spots of different radii. Data from four experiments are shown as mean ± SEM. Values from one experiment all have the same symbol.
However, $D$ was independent of Qa-2 concentration, measured as fluorescence intensity, even when small regions were probed, using a 0.4- or 0.6-μm spots (data not shown).

The results on Qa-2 antigens suggest that the transmembrane domain and/or the cytoplasmic tail of H-2D$^b$ keeps it confined to domains. To test this we measured the lateral diffusion of two hybrid molecules expressed on the surface of HEPA-OVA cells after transfection with the appropriately modified class I MHC genes. Fig. 1 diagrams the predicted domain organization of the two hybrids. The hybrid expressed in K123/12 consists of the exodomains of the Qa-2 molecule joined to the transmembrane and cytoplasmic domains with the sequence of the conventional H-2 molecule, H-2D$^b$. The hybrid expressed in K123/13 consists of the first two external domains, alpha-1 and alpha-2 of the Qa-2 molecule, the third external domain, alpha 3, with the sequence of H-2D$^b$ and the GPI membrane linkage specified by a portion of the Q7 gene. Fig. 7, a and b compares the distributions of mobile fractions of the construct molecule, K123/12, peptide linked to the bilayer, measured with 0.6- and 1.1-μm-radius spots. The mobile fraction of this molecule is significantly larger when measured with the 0.6-μm spot than when measured with the 1.1-μm spot ($R = 0.50 \pm 0.08$ vs. $R = 0.37 \pm 0.11$). On the other hand, the mobile fraction of the GPI-linked construct, K123/13, is no different when measured with the 0.6-μm spot than when measured using the 1.1-μm spot ($R = 0.42 \pm 0.16$ vs. $R = 0.46 \pm 14$, respectively) (Fig. 7, c and d). $D$ of both constructs was $\sim 5 \times 10^{-10}$ cm$^2$·s$^{-1}$ when measured with the 0.6-μm spot and $\sim 2 \times 10^{-10}$ cm$^2$·s$^{-1}$ when measured with the 1.1-μm spot.

**Discussion**

We have previously shown that the cell surface of fibroblast membranes contains membrane domains experimentally defined by the dependence of the mobile fraction, $R$, and the diffusion coefficient, $D$, of a fluorescent lipid analogue, NBD-PC on the size of the laser spot used for FPR measurements of lateral diffusion. The mobile fraction decreased with increasing spot size, while $D$ increased with increasing spot size. These results, together with fluorescence images of labeled cell surfaces, led to a model of membrane domains rich in membrane proteins, separated by regions relatively poor in proteins. FPR measurements of membrane proteins labeled with fluorescent Fab fragments also showed a dependence of both $R$ and $D$ on spot size.

The data presented here extend our operational definition of membrane domains to the surface of K78-2, mouse hepatoma, cells. $R$ and $D$ of NBD-PC in these cells show a dependence on spot size as they did in human fibroblast membranes over a range of laser spot radii from 0.3 to 2.0 μm. In this range $R$ varied from $\sim 0.8$ to $\sim 0.25$ and $D$ varied from $\sim 1 \times 10^{-10}$ to $\sim 25 \times 10^{-10}$ cm$^2$·s$^{-1}$. As is the case in fibroblast membranes another fluorescent lipid analog, the indocarbocyanine dye diI C16, does not show this behavior. The relatively small difference in $D$ of diI C16 measured at two different spot sizes could be largely accounted for by small errors (10-15%) in measurement of the laser beam radius.

Two different class I MHC molecules are expressed at the surface of K78-2 hepatoma cells. One, an endogenous class I molecule, H-2D$^d$, is anchored to the membrane bilayer through a transmembrane peptide of 23 residues; a cytoplasmic tail of 27 residues follows this transmembrane domain. The other class I molecule, Qa-2, the product of a transfected Q7 gene, is >80% homologous to H-2D$^b$, but is anchored to the membrane through a phosphatidylinositol glycan, GPI, which only extends into the outer leaflet of the bilayer. The expression of the two homologous proteins in one cell membrane resolves the effects of a GPI anchorage on lateral diffusion behavior in a way that has not been possible in published measurements of the lateral diffusion of GPI-linked proteins (Cowan et al., 1987; Ishihara et al., 1987; Phelps et al., 1988). Though membrane anchorage by GPI is not reflected in differences between the diffusion coefficients of the H-2D$^d$ and Qa-2 molecules, it is evident in the ways in which the molecules are constrained in their lateral mobility. The apparent mobile fraction, $R$, of the peptide-anchored H-2D$^d$ molecule depends upon the size of the laser spot.

![Figure 6](https://example.com/figure6.png)

Figure 6. The mobile fractions of Fl-Fab-labeled Qa2 molecules in K78 hepatoma cell membranes when measured with laser spots of different radii. Data from two experiments are shown as mean ± SEM. Values from one experiment all have the same symbol.

![Figure 7](https://example.com/figure7.png)

Figure 7. The mobile fractions, measured with laser spots of different radii, of the transmembrane peptide–anchored Qa-2 molecule expressed in K123-12-4 hepatoma cells (a and b). (a) Spot radius, 0.6 μm (40× objective). Average mobile fraction is 0.50 ± 0.08. $D = 4 \times 10^{-10}$ cm$^2$·s$^{-1}$. (b) Spot radius, 1.1 μm (22× objective). Average mobile fraction is 0.37 ± 0.11. $D = 25 \times 10^{-10}$ cm$^2$·s$^{-1}$. The GPI-anchored Qa-2/H-2D hybrid molecule expressed in K123-13-4 hepatoma cells (c and d). (c) Spot radius, 0.6 μm (40× objective). Average mobile fraction is 0.42 ± 0.10. $D = 8 \times 10^{-10}$ cm$^2$·s$^{-1}$. (d) Spot radius, 1.1 μm (22× objective). Average mobile fraction is 0.46 ± 0.14. $D = 21 \times 10^{-10}$ cm$^2$·s$^{-1}$. 

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used in an FPR measurement, while the mobile fraction of the GPI-anchored protein is independent of spot size. These relationships between spot size and $R$ are also true for hybrid class I molecules. A hybrid, K123/12, with the exodomain of Qa-2 and transmembrane and cytoplasmic regions of a conventional class I antigen, H-2D$^b$, appears to be constrained by membrane domains, while a hybrid, K123/13, with a mixture of Qa-2 and H-2D$^b$ domains terminating in the GPI membrane linkage, behaves like the native Qa-2 molecule. $R$ does not change with changes in laser spot size.

The diffusion coefficients of both H-2D$^b$ and Qa-2 increased with increasing spot size. However, for a given small spot size $D$ for H-2D$^b$ was low when fluorescence intensity, a measure of protein concentration, was high and vice versa while $D$ for Qa-2 was not correlated at all with fluorescence intensity.

These results bear on three points: the nature of the regions probed by the NBD-lipid analogue, the locus of constraints to the lateral diffusion of membrane proteins H-2D$^b$ and Qa-2, and the structural basis of the membrane domains defined by our FPR experiments.

In our hands the diffusibility of NBD-PC added to cells by injection of a few microliters of stock solution in ethanol has consistently resembled that of proteins, rather than that of native lipids. In particular $D$ measured with our usual small laser spot, radius 0.6–0.8 $\mu$m, is in the range of $1-3 \times 10^{-10} \text{cm}^2 \cdot \text{s}^{-1}$, similar to that for many membrane proteins. It is not likely that this behavior is due to the partition of the lipid analogue into gel domains since this class of lipid analogues partitions into fluid phases due to its high water solubility. It may be that this lipid analogue associates with membrane proteins to a greater extent than do lipid analogues such as the indocarbocyanines whose hydrophobic regions are unmodified and which can selectively partition into gel or fluid phases. The capping of NBD-PC reported earlier is consistent with this idea [Schroit and Pagano, 1981]. If this is so then the spatial differentiation of membranes reported by measurements of the lateral diffusion of NBD-PC involves both proteins and lipids and may be dominated by lipid/protein, rather than lipid/lipid, interactions.

The diffusion coefficients, $D$ measured for H-2D$^b$ and Qa-2 molecules are comparable for all laser spot sizes used. They are low, in the range $1-5 \times 10^{-10} \text{cm}^2 \cdot \text{s}^{-1}$, for spots of small area, and are $5-20$ times larger, in the range $1-30 \times 10^{-10} \text{cm}^2 \cdot \text{s}^{-1}$, when large spots are used. The higher values in this range approach $D$ for viscosity-limited diffusion of proteins in lipid bilayers, $50-100 \times 10^{-10} \text{cm}^2 \cdot \text{s}^{-1}$. We have previously shown that in some instances $D$ for membrane glycoproteins is largely constrained by interactions between proteins in the plane of the membrane, and not by interactions with the cytoskeleton (Wier and Edidin, 1988). The results here, in which molecules with and without cytoplasmic domains have the same values of $D$ are also consistent with this interpretation.

The changes in apparent $D$ with increasing spot size cover an even greater range (20–25 times) than those that we observed previously for membrane proteins (Yechiel and Edidin, 1987). This range may reflect the biology and organization of K78-2 surfaces, but it could to some extent be due to any or all of a number of optical artifacts: improper measurement of beam radii produced by different objectives, focusing errors (especially with small spots), slight misalignment of the spot with the aperture used to limit light collection by the photomultiplier, and failure of the electronics to resolve rapid recoveries of fluorescence in very small spots. It is unlikely that the first of these contributes significantly to the observed range of $D$. Our measurements of spot size analyze an expanded image of the beam following a published procedure (Schneider and Webb, 1981) and are sufficiently accurate so that $D$, calculated from $t_{1/2}$, of fluorescent IgG in 98% glycerol solution, is quite similar for all spot sizes examined and is within 20% of the value calculated from published values for IgG diffusion in water and the measured viscosity of the glycerol solution. Critical focusing, though difficult, is easiest for high NA, high magnification, objectives; hence, it is unlikely to produce gross errors, of the magnitude (about threefold) required to give the observed range of $D$. Judging from some published work, a defocus of $\sim 2 \mu$m would be required to increase the spot size threefold (Hiraoka et al., 1990).

The third and fourth artifacts mentioned may be important. In model systems we can see clearly that misalignment of spot to aperture can cause a lag in the observed recovery. Such a lag could mask rapid recoveries. Alignment of the spots is checked daily and each time the spot size is changed. It is unlikely to be a major contributor to our results. Resolution of very fast recoveries is potentially a great problem for our results. However, we have found that the smallest spots that we can produce, 0.35–0.45 $\mu$m, can resolve recoveries when $D$ is $< 2 \times 10^{-9} \text{cm}^2 \cdot \text{s}^{-1}$ and slightly larger spots, 0.6–0.7 $\mu$m, can resolve recoveries when $D$ is $\sim 5 \times 10^{-9} \text{cm}^2 \cdot \text{s}^{-1}$. Hence, it is likely that the $D$ measured with small spots reflects membrane biology rather than being due to optical artifacts. Another group have also found that $D$ of a membrane protein measured with small spots (0.8 $\mu$m radius) is five- to sixfold smaller than $D$ measured with spots of 2–4 $\mu$m radius [Dubinsky et al., 1989]. This group suggested that recoveries on short time scales are dominated by

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**Figure 8.** A schematic model of membrane domains. Illuminated areas, which may be larger or smaller than the area of a domain, are shaded. Domains are indicated as circles. $D_1 (< D_2)$ in the bounded, protein-rich domains and $D_2$ in the relatively lipid region between the domains.
the times of interaction of the diffusing molecules with constraints; either immobile membrane proteins or proteins of the cytoplasm.

The mobile fraction of Qa-2 was ~0.5 for all measuring spot sizes and was not changed by interferon treatment of the cells; the mobile fraction of H-2D\* was never higher than ~0.6. Though the low apparent mobile fraction is due in part to a small fraction of nonspecifically bound fluorescence and to cellular autofluorescence (both of which bleach but do not recover), most of it is due to specifically labeled class I molecules that are immobile on the time scale of an FPR experiment. Similar immobile fractions, from 20 to 70% of a population of molecules are found for other inositol lipid-linked proteins as well as for peptide-anchored membrane proteins (reviewed in Edidin, 1990b).

Our most important experimental criteria for locating a molecule in membrane domains are the relationship between \( R \) and spot size, and the relationship between \( D \) and concentration of labeled molecules. The results obtained on H-2D\* molecules fit these criteria. \( R \) decreases with increasing spot size, and for small spots \( D \) is lower in spots of higher protein concentration than in spots of lower protein concentration (measured as fluorescence intensity). These relationships are consistent with our previous model in which proteins are enriched in membrane domains of micrometer scale which are surrounded by a continuum of relatively protein-poor membrane (Fig. 8). The results on Qa-2 molecules do not fit our criteria, but they lead to further definition of the model of Fig. 8. Since the main difference between H-2D\* and Qa-2 is in their mode of anchorage to the bilayer and the extent of their interaction with the cell cytoplasm we propose that the domains detected in our experiments arise from interactions of membrane proteins with cell cytoskeleton. Further experimental evidence for the importance of the cytoplasmic tail in concentrating proteins in domains comes from our results on mutant proteins. Molecules with the external portion of Qa-2 but linked to the bilayer by a transmembrane peptide with a cytoplasmic tail appear by our criteria to be concentrated in domains. Hybrid class I molecules with a significant portion derived from a peptide-linked, classical, class I antigen, but linked to the bilayer by GPI do not appear to be concentrated in membrane domains. It appears that proteins without a cytoplasmic tail cross the boundaries of membrane domains more readily than molecules carrying transmembrane and cytoplasmic segments. However, diffusion of GPI-linked proteins within membrane domains is impeded by a high concentration of membrane proteins.

On this interpretation \( D \) for Qa-2 ought to be a mixture of \( D_1 \), for diffusion within a protein-rich domain, and \( D_2 \), for diffusion in the continuum, relatively poor in proteins, between the domains. If the area of the domains is greater than that of the continuum between them, then most FPR measurements with small laser spots will give recovery curves dominated by \( D_1 \). Small spots falling on regions outside the domains \( D = D_2 \) will result in truncated recovery curves if \( D_2 \) is \( >2 \times 10^{-3} \text{ cm}^2 \cdot \text{s}^{-1} \). Measurements using large spots will give recoveries dominated by \( D_2 \), diffusion of Qa-2 molecules in the continuum, but molecules diffusing at \( D_1 \), within domains will also contribute. The mixture of recoveries will underestimate \( D_2 \) and the total recovery. The extent of the error depends upon the magnitudes of \( D_1 \) and \( D_2 \) and on their fractional contributions, \( p_1 \) and \( p_2 \), to the recovery curve, but it appears that even a large fraction of \( D_1 \) will have a small effect. For example, let us take \( D_2 = 5 \times 10^{-4} \text{ cm}^2 \cdot \text{s}^{-1} \), \( p_2 = 0.33 \); \( D_1 = 2.5 \times 10^{-10} \text{ cm}^2 \cdot \text{s}^{-1} \); \( p_1 = 0.67 \) and a spot radius, \( w = 1.3 \mu \text{m} \). With these values of \( w \) and \( D_2 \), \( t_{1/2} \) is ~1 s and one-third of the total recovery will occur in ~5 s. The rest of the recovery will be due to molecules diffusing at \( D_1 \). If \( t_{1/2} \) for this recovery is ~20 s. Very roughly the total recovery (with contributions by both populations of molecules) will be 45% of maximum at 5 s, 50% at 10 s, 67% at 20 s, and 83% at 40 s. The fit of such a curve by our program would be close to the fit for a single component recovery.

Our results extend the experimental definition of membrane domains previously made for proteins of human fibroblasts to class I proteins of hepatoma cells. They further suggest that the basis for these domains lies in the inner leaflet of the bilayer or in the cell cytoplasm. The scale of the domains appears to be in micrometers. Such a scale could be imposed by the spacing of stress fibers in fibroblasts [for example see Bershadsky and Vasiliev, 1988] or by a spectrin (fodrin) cytoskeleton. The mesh of spectrin tetramers observed in the erythrocytes has dimensions of ~0.2 \( \mu \text{m} \) per side (Shen et al., 1986; Liu et al., 1987). Imperfect meshes or patches of mesh separated by spectrin-free regions could form the basis for the organization of cell surfaces into domains. If such patches are coupled to the lipid bilayer and exert lateral pressure on the bilayer lipids they could also serve to induce lipid/lipid immiscibilities that are the basis for other sorts of membrane domains, for example those detected by the indocarbocyanine dyes such as diIC16. Both protein diffusibility and lipid domain formation would be modulated by fluctuations in the spectrin assembly (Saxton, 1990).

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