Measurement of the Lateral Diffusion of Human MHC Class I Molecules on HeLa Cells by Fluorescence Recovery after Photobleaching Using a Phycoerythrin Probe

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ABSTRACT The mobility of cell surface MHC class I molecules on HeLa cells was measured by fluorescence recovery after photobleaching (FRAP). The probe used for these studies was the phycobiliprotein R-phycoerythrin coupled to Fab fragments of a monoclonal antibody specific for human monomorphic MHC class I molecules. It was found that the recovery curves could be equally well fitted by either a random diffusion model with an immobile component or by an anomalous diffusion model. In the latter case, the anomalous diffusion exponent was consistent with that previously determined by single-particle tracking (SPT) experiments using the same probe (P. R. Smith, I. E. G. Morrison, K. M. Wilson, N. Fernandez, and R. J. Cherry. 1999. *Biophys. J.* 76:3331–3344). The FRAP experiments, however, yielded a considerably higher value of D_0, the diffusion coefficient for a time interval of 1 s. To determine whether the results were probe dependent, FRAP measurements were also performed with the same monoclonal antibody labeled with Oregon Green. These experiments gave similar results to those obtained with the phycoerythrin probe. FRAP experiments with the lipid probe 5-*N*-(octadecanoyl) aminofluoroscein (ODAF) bound to HeLa cells gave typical results for lipid diffusion. Overall, our observations and analysis are consistent with anomalous diffusion of MHC class I diffusion on HeLa cells, but quantitative differences between FRAP and SPT data remain to be explained.

INTRODUCTION

Lateral diffusion in cell membranes is currently measured by two principal methods: fluorescence recovery after photobleaching (FRAP) and single-particle tracking (SPT) (Jovin and Vaz, 1989; Peters and Scholz, 1991, Zhang et al., 1993; Saxton and Jacobson, 1997; Kusumi and Sako, 1996). FRAP measures the average diffusion of a population of molecules over distances typically of the order of 1 μm. SPT permits the observation of the movements of single molecules with a spatial resolution of typically 10–20 nm. FRAP data are normally analyzed by a model that incorporates a randomly diffusing mobile fraction and an immobile fraction. Variation of the size of the photobleached area may provide evidence that molecules are constrained within domains (Edidin and Stroynowski, 1991; Schram et al., 1994; Salome et al., 1998). Feder et al. (1996) applied an anomalous diffusion model to the interpretation of FRAP experiments. They showed that in some, and probably most, cases the FRAP data can be fitted equally well by this model as by the conventional model. Anomalous diffusion in cell membranes may result from obstacles or traps with a broad distribution of binding energies or escape times (Saxton, 1996).

SPT measurements have now been performed with a number of receptors on different cell types (Saxton and Jacobson, 1997). In all cases so far investigated, departures from simple diffusion have been detected. In addition to random motion, both directed motion and constrained diffusion have been observed, and in some cases all three types of motion are apparently present on the same cell (Kusumi et al., 1993; Wilson et al., 1996; Simson et al., 1998). In the case of constrained diffusion, two different interpretations of the phenomenon have emerged. In one model, receptors move randomly within sub-micrometer domains, their long-range diffusion determined by the rate at which they can escape from these domains (Sako and Kusumi, 1994, 1995; Kusumi and Sako, 1996). In a related model, receptors undergo random diffusion interspersed with periods of temporary confinement (Simson et al., 1995, 1998).

We recently reported the results of a detailed study by SPT of the mobility of MHC class I molecules on Hela cells (Smith et al., 1999). MHC class I molecules were labeled using the Fab fragment of a monoclonal antibody covalently bound to R-phycoerythrin (PhyE) and the particles tracked using high-sensitivity fluorescence imaging. Analysis of the data for a fixed time interval suggested a reasonable fit to a random diffusion model. The best-fit values of the diffusion coefficient D decreased markedly, however, with increasing time interval, demonstrating the existence of anomalous sub-diffusion. Further analysis of the data showed that diffusion is anomalous over the complete time range investigated, 4–300 s.

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In view of the considerable differences in the way that mobility is measured by FRAP and by SPT, we thought it would be of value to compare the two techniques. Previous comparisons of FRAP and SPT are generally complicated by the use of different probes for the two different measurements. The R-phycoerythrin (PhyE) probe that we used for SPT can, however, also be employed for FRAP measurements. We have therefore performed a FRAP study of the mobility of MHC class I molecules on HeLA cells under conditions that are essentially identical to those used for the SPT experiments. We find that the FRAP data can be accounted for by an anomalous diffusion model with an anomalous diffusion exponent in reasonable agreement with that obtained by SPT. There is, however, an unexplained discrepancy between the magnitudes of the diffusion coefficients obtained from the two techniques.

MATERIALS AND METHODS

Cells and antibodies

HeLa cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with fetal calf serum (FCS) (10% v/v), glutamine (2 mM), and streptomycin/ampicillin at 37°C in a humidified atmosphere of 7% CO_2. Trypsinized cells were seeded into eight-well LabTek chambers (Gibco) (5×10^3 cells per well) and cultured for 72 h before imaging. IgG was purified from ascites fluid (obtained from the Royal London Hospital Medical College) containing W6/32 (a pan-reactive, class-I-specific, monoclonal antibody) using a protein G HiTrap column (Pharmacia, St. Albans, UK). Fab fragments were prepared by papain digestion as described previously (Smith et al., 1998).

Preparation and HPLC purification of an R-phycoerythrin-Fab conjugate (PhyE-Fab)

Fab fragments were purified by size-exclusion HPLC (using a Bioselect Sec 250–5 size exclusion column obtained from Biorad, Hemel, Hempstead, UK) and conjugated with the pyridyl disulfide derivative of PhyE (Molecular Probes, Eugene, OR) as described previously (Smith et al., 1998; Triantafilou et al., 2000). Briefly, Fab fragments were dialyzed against sodium phosphate buffer (20 mM, pH 7.0) containing NaCl (0.1 M) and concentrated to 5 mg ml^{-1} using a Centristart 1 device (10-kDa exclusion limit obtained from Sartorius, Göttingen, Germany). Ten molar equivalents of a stock solution of succinimidyl *trans*-4-(*N*-maleimidyl-methyl)cyclohexane-1-carboxylate (5 mM stock in dimethylsulfoxide (DMSO)) were added to the Fab and incubated for 2 h at room temperature. Excess succinimidyl *trans*-4-(*N*-maleimidyl-methyl)cyclohexane-1-carboxylate was removed by extensive dialysis against phosphate buffer. In parallel, the pyridyl disulfide derivative of PhyE (1 mg, average 1.6 pyridyl disulfide derivatives per molecule) was incubated with dithiothreitol (DTT) (4 mg) for 15 min at room temperature, in the dark. Excess DTT was removed by extensive dialysis against phosphate buffer. The PhyE was then incubated with the Fab for 20 h at 4°C in the dark. Further reaction was stopped by the addition of a 20 times excess of *N*-ethylmaleimide (Sigma, Poole, UK). PhyE and its conjugates were always handled in the dark to avoid photobleaching.

The PhyE-Fab was purified by size-exclusion chromatography on a BioRad 5000T HPLC. PhyE-Fab (300 μl) was loaded onto a Bio-Select SEC 250–5 column equilibrated with phosphate buffer (20 mM, pH 7.0) containing NaCl (0.1 M) and eluted at 0.1 ml min^{-1}. Fractions (100 μl) were collected and analyzed for activity by flow cytometry. Integration was performed using ValueChrom integration analysis software (BioRad). Specific binding activity of the probe was checked by flow cytometry as previously described (Smith et al., 1999; Triantafilou et al., 2000).

Preparation of Oregon Green 514-IgG conjugate (OG-IgG)

Oregon Green 514 carboxylic acid, succinimidyl ester (OG514), was obtained from Molecular Probes. For an ~4:1 probe:protein ratio, OG514 was dissolved in DMSO at a concentration of 10 mg/ml immediately before starting the conjugation. The labeling mixture consisted of 300 μl of the purified IgG (0.7 mg/ml), 5 μl of the probe solution, and 30 μl of 1 M sodium bicarbonate, pH 8.3. The probe was added to the IgG and sodium bicarbonate while slowly vortexing, and the reaction mixture allowed to incubate in the dark for 1 h at room temperature. The reaction was then terminated by the addition of 10% v/v hydroxylamine (1.5 M, pH 8.5). Unbound probe was removed by passing the mixture down a PD10 column (Sephadex G25, Pharmacia) in the dark and collecting the first eluted band that corresponded to the conjugate. The probe:protein ratio was determined by calculating the concentration of the OG514 from the absorbance at 509 nm using the Beer-Lambert equation (extinction coefficient for OG514 is 85,000 M^{-1} cm^{-1}), and the concentration of the IgG was measured using the Bradford assay (Bradford, 1976).

Cell labeling with fluorescent antibodies

HeLa cells were seeded onto LabTek slides (Gibco) at a density of 5000 cells/well and cultured for 72 h before imaging. The cells were gently washed twice with PBS and then incubated with PhyE-Fab or OG514-IgG in PBS for 30 min at room temperature in the dark. The cells were gently washed at least five times with PBS, sealed with a coverslip, and transferred to a microscope stage maintained at 22°C.

Cell labeling with ODAF (5-N-(octadecanoyl) aminofluoroscein)

Cells grown on LabTek slides were incubated on ice in the dark with PBS containing 2 μM ODAF (Molecular Probes) for 15 min. The cells were then washed with fresh PBS and sealed with a coverslip as described above.

FRAP measurements

FRAP measurements were performed as previously described (Ladha et al., 1994, 1996). Briefly, PhyE and OG514 were excited at 514 nm and ODAF at 488 nm using a laser beam of Gaussian cross-sectional intensity. The half-width at $1/e^2$ height of the laser beam at its point of focus was equal to either 1.24-μm or 2.15-μm spot radius. The beam was generated by a water-cooled argon ion laser, bleaching powers were 0.2–0.4 W, and bleaching times were 5–50 ms. Recovery curves were analyzed as previously described (Ladha et al., 1994, Yguerabide et al., 1982) to obtain a mobile fraction characterized by a diffusion coefficient D and an immobile fraction.

The FRAP recovery curves were also analyzed for anomalous diffusion by assuming a time-dependent diffusion coefficient and no immobile fraction as described by Feder et al. (1996). D is then given by:

$$D = D_0 t^{\alpha-1} \quad (1)$$

where D_0 is a constant (the value of D at $t = 1$ s) and α is the anomalous diffusion exponent.

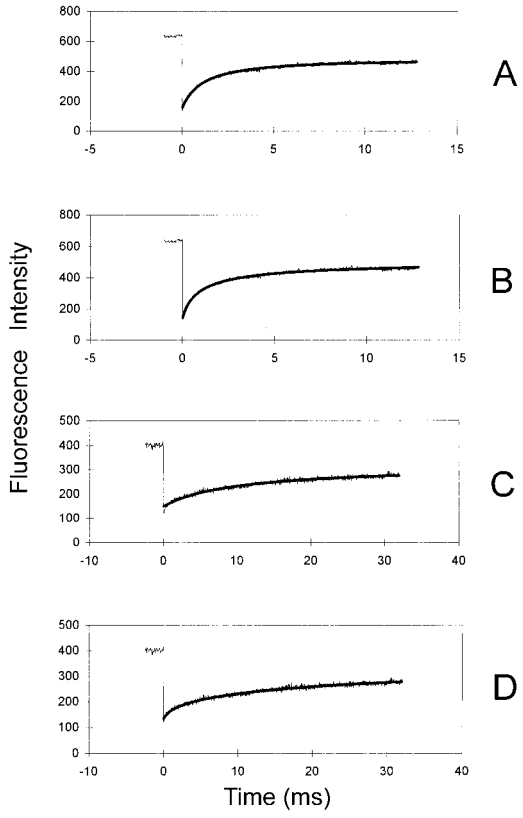

FIGURE 1 Averaged FRAP curves for PhyE-Fab-labeled MHC class I molecules on HeLa cells measured at 22°C. (*A*) 1.24-μm spot size, data fitted by random diffusion with immobile fraction; (*B*) 1.24-μm spot size, data fitted by anomalous diffusion; (*C*) 2.15-μm spot size, data fitted by random diffusion with immobile fraction; (*D*) 2.15-μm spot size, data fitted by anomalous diffusion.

RESULTS

Fig. 1 shows typical results of FRAP experiments performed with HeLa cells labeled with PhyE-Fab bound to MHC class I molecules. Experiments were performed with two different sizes of the bleached spot. The recovery curves were fitted either by the standard model of random diffusion plus an immobile fraction or by the anomalous diffusion model as described in Materials and Methods. The two models generally gave equally good fits to the data, as judged by the values of χ^2. The results of between 10 and 35 individual FRAP curves obtained for different cells on the same microscope slide were averaged before fitting. The experiments were repeated several times using freshly prepared microscope slides and also on different days. The results of different experiments are collected together in Table 1. In addition to fitting the averaged FRAP curves, individual curves were also fitted to determine cell-to-cell variability. The results are illustrated in Figs. 2 and 3. In a few cases, we measured lateral diffusion on different areas of the same cell. We found that the variability over the cell surface was comparable to cell-to-cell variability.

Similar FRAP experiments were performed with IgG labeled with OG514. The averaged data were fitted by the standard model of random diffusion plus an immobile fraction (Fig. 4). The parameters obtained are given in Table 1. The cell-to-cell variability obtained from fitting individual FRAP curves is shown in Figs. 2 and 3. Fits to the anomalous diffusion model were not possible because the parameters failed to converge.

In experiments with both PhyE-Fab and OG514-IgG, the prebleach signal was quite variable, indicating varying labeling densities in the illuminated spot. To determine whether the labeling density has any effect on the diffusion parameters, the diffusion coefficients and mobile fractions were plotted against the prebleach fluorescence intensity. As can be seen in Figs. 5 and 6, there is no evidence in most cases for a correlation between prebleach intensity and either the diffusion coefficient or the mobile fraction. In the

TABLE 1 Analysis of FRAP data for HeLa cells

		Immobile fraction		Anomalous diffusion	
Probe	Spot size (μm)	D (cm^2 s^{-1} $\times$ 10^9)	R (%)	D_o (cm^2 s^{-1} $\times$ 10^9)	α
PhyE-Fab	1.24	1.7±0.1	72	2.7±0.6	0.51±0.02
PhyE-Fab	1.24	4.9±0.3	70	6.4±0.2	0.42±0.02
PhyE-Fab	1.24	3.4±0.3	66	3.7±0.1	0.43±0.02
PhyE-Fab	1.24	1.7±0.2	57	2.7±0.3	0.36±0.02
PhyE-Fab	2.15	1.5±0.1	66	2.2±0.2	0.59±0.03
PhyE-Fab	2.15	1.8±0.2	67	2.6±0.2	0.58±0.03
PhyE-Fab	2.15	1.7±0.1	62	2.2±0.2	0.57±0.02
PhyE-Fab	2.15	1.8±0.1	76	2.3±0.1	0.69±0.02
PhyE-Fab	2.15	2.2±0.1	71	2.8±0.1	0.63±0.01
PhyE-Fab	2.15	1.4±0.1	64	1.8±0.1	0.62±0.02
OG-IgG	1.24	1.7±0.2	55		
Og-IgG	2.15	1.3±0.1	51		
ODAF	1.24	11 ± 1	82		

Each row corresponds to a separate experiment in which 10–35 individual FRAP curves were averaged and fitted. The data were fitted by (a) random diffusion with diffusion coefficient D plus an immobile component (R is the percentage recovery of the prebleach fluorescence intensity) and (b) anomalous diffusion characterised by D_0 (the value of D at t = 1 s) and α, the anomalous diffusion exponent. Experiments performed at 22°C.

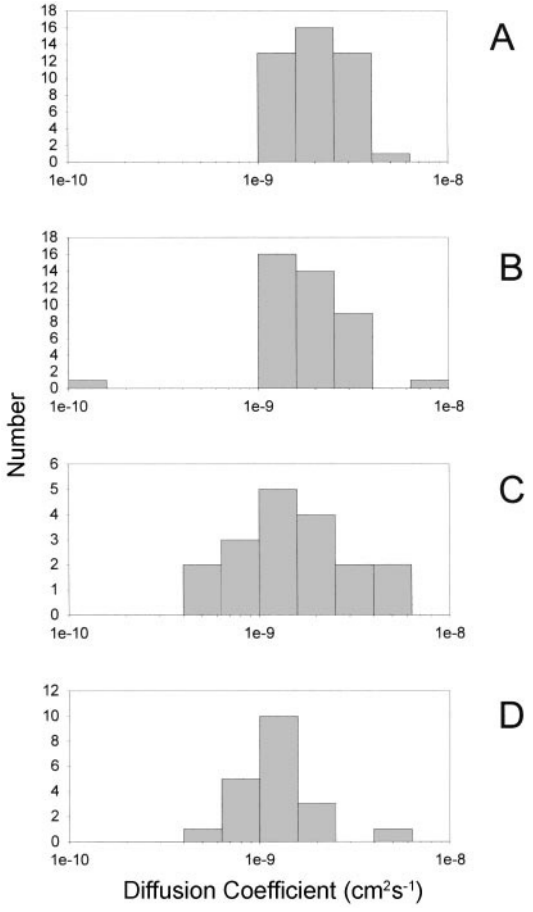

FIGURE 2 Cell-to-cell variation of D. Individual FRAP curves on different cells were fitted by random diffusion with immobile fraction. (*A*) PhyE-Fab probe, 1.24-μm spot; (*B*) PhyE-Fab probe, 2.15-μm spot; (*C*) OG-IgG probe, 1.24-μm spot; (*D*) OG-IgG probe, 2.15-μm spot.

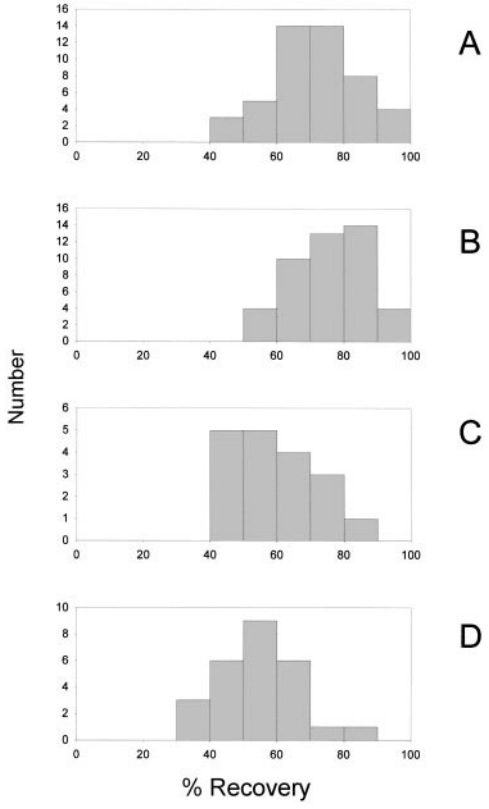

FIGURE 3 Cell-to-cell variation of percent R. Individual FRAP curves on different cells were fitted by random diffusion with immobile fraction. (*A*) PhyE-Fab probe, 1.24-μm spot; (*B*) PhyE-Fab probe, 2.15-μm spot; (*C*) OG-IgG probe, 1.24-μm spot; (*D*) OG-IgG probe, 2.15-μm spot.

case of OG514-IgG there is possibly a slight decrease in the mobile fraction at the higher prebleach intensities.

The lipid probe ODAF was also used to measure lipid diffusion in HeLa cells. The results of these experiments are included in Table 1.

DISCUSSION

Phycobiliproteins are not normally used as probes for FRAP experiments. They were employed in the current study to permit a comparison with the results of SPT experiments. An advantage of a probe such as PhyE is that it exhibits brighter fluorescence than can be obtained with fluorescent-labeled antibodies. This could be helpful for FRAP studies of molecules present in low abundance, although this is not a factor in the present experiments with MHC class I molecules.

The FRAP results that we obtain for PhyE-Fab bound to MHC class I molecules on HeLa cells are in line with those typically obtained with cell surface receptors. We find that the FRAP curves can be fitted by the standard model of a mobile, randomly diffusing population plus an immobile population. There is considerable cell-to-cell variation, as can be seen in Figs. 2 and 3. The average value of the

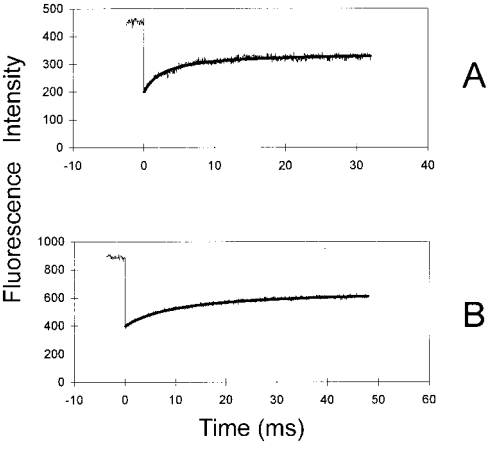

FIGURE 4 Averaged FRAP curves for OG-IgG-labeled MHC class I molecules on HeLa cells measured at 22°C. (*A*) 1.24-μm spot size, data fitted by random diffusion with immobile fraction; (*B*) 2.15-μm spot size, data fitted by random diffusion with immobile fraction.

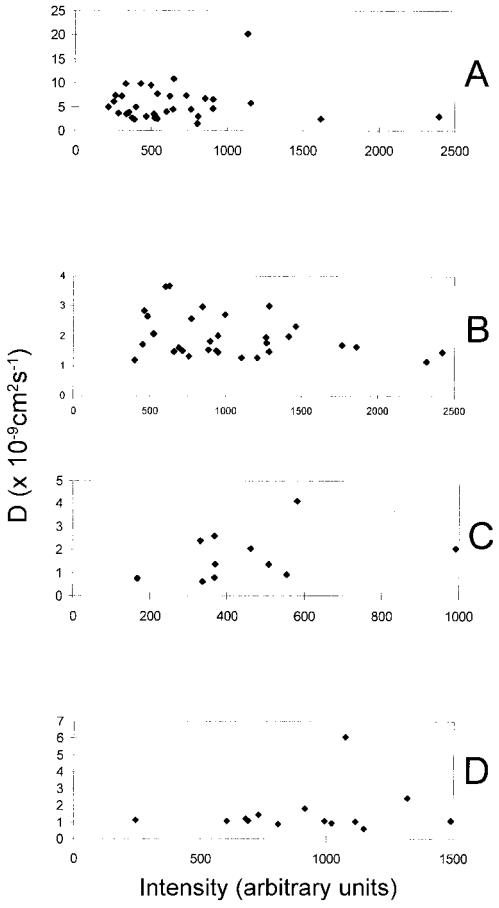

FIGURE 5 Scatter plot of D against prebleach intensity. Each individual FRAP recording was fitted by random diffusion plus an immobile fraction. (*A*) PhyE-Fab probe, 1.24-μm spot; (*B*) PhyE-Fab probe, 2.15-μm spot; (*C*) OG-IgG probe, 1.24-μm spot; (*D*) OG-IgG probe, 2.15-μm spot.

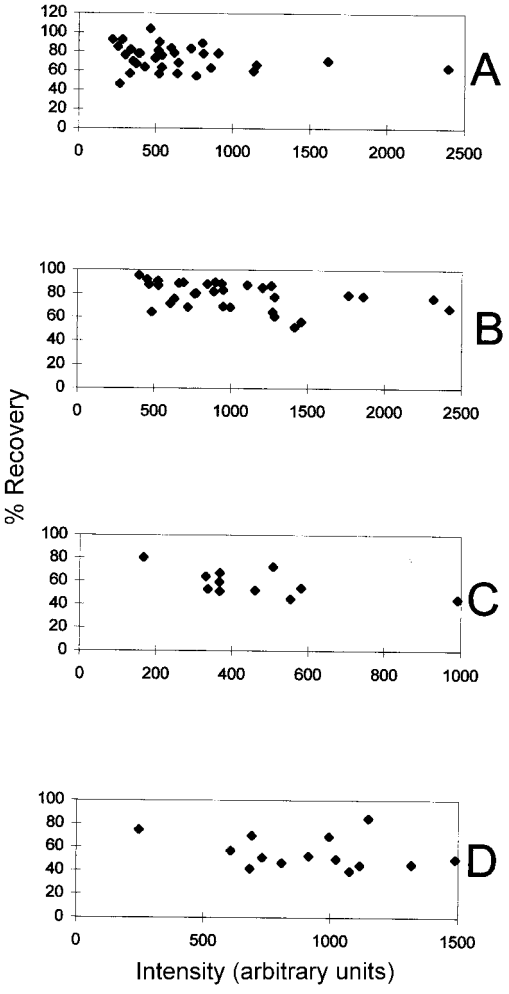

FIGURE 6 Scatter plot of percent R against prebleach intensity. Each individual FRAP recording was fitted by random diffusion plus an immobile fraction. (*A*) PhyE-Fab probe, 1.24-μm spot; (*B*) PhyE-Fab probe, 2.15-μm spot; (*C*) OG-IgG probe, 1.24-μm spot; (*D*) OG-IgG probe, 2.15-μm spot.

diffusion coefficient is on the order of (1–3) $\times$ 10^{-9} cm^2 s^{-1}, toward the upper end of typical FRAP values for proteins in cell membranes. The mobile fraction is 60–70%, which is within the range commonly encountered for cell surface molecules. A decrease in the mobile fraction with increasing spot size may indicate the presence of membrane domains (Edidin and Stroynowski, 1991; Salome et al., 1998). Within experimental error, however, the mobile fraction was the same for the two spot sizes employed.

We also performed FRAP experiments on HeLa cells with the lipid probe ODAF. These gave diffusion coefficients of $\sim$1 $\times$ 10^{-8} cm^2 s^{-1}, typical for free lipid diffusion (Jovin and Vaz, 1989). The mobile fraction was $\sim$80%. Although 100% recoveries might be expected for lipid diffusion, lower values are not unusual (Edidin and Stroynowski, 1991) and may reflect partial entrapment in domains.

Feder et al. (1996) previously showed that FRAP curves may also be fitted by an anomalous diffusion model. In this model, the failure of the fluorescence to exhibit 100% recovery is due to the decrease in diffusion coefficient with time and the limited duration of the measurement. We also find that the present data for the PhyE-Fab probe can be equally well fitted by the anomalous diffusion model.

A major aim of these studies was to compare the results of FRAP and SPT measurements. We recently reported the results of an SPT study performed with the same PhyE-Fab probe directed against MHC class I molecules on HeLa cells under essentially identical conditions to the FRAP studies presented here (Smith et al., 1999). We found strong evidence for anomalous subdiffusion over the time range 4–300 s with an anomalous diffusion exponent of 0.49 $\pm$ 0.16. In these experiments, we observed no MHC class I molecules that were completely immobile. In agreement with these results, we find that the FRAP data are well fitted by an anomalous diffusion model. The anomalous diffusion exponents shown in Table 1 are consistent with the SPT

values. In so far as no immobile molecules were observed with SPT, the standard FRAP model is not consistent with SPT although the fits to the data are equally good. Feder et al. (1996) previously pointed out that curve fitting was unlikely to distinguish between the two interpretations of FRAP in most cases.

The structural basis of anomalous diffusion or other constraints on the mobility of cell membrane components is not at all clear in most cases. There is, however, a considerable amount of evidence for the involvement of cytoskeletal elements (Saxton and Jacobson, 1997; Kusumi and Sako, 1996). Our finding that the FRAP data are similar for two different sized probes bound to the extracellular surface also suggests that the principal constraints are not extracellular. Edidin et al. (1994) previously found evidence of cytoplasmic barriers to diffusion of murine MHC class I molecules on mouse hepatoma cells by observing the movements of mutants truncated in their cytoplasmic domain.

Although anomalous diffusion appears to provide an adequate explanation of both the SPT and FRAP data, there is a significant discrepancy between the values of D_0, the value of the diffusion coefficient over 1 s. The SPT experiments yielded a value of $(6.7 \pm 4.5) \times 10^{-11}$ cm^2 s^{-1}, about one to two orders of magnitude lower than the FRAP values in Table 1. The only experimental difference between the samples used for FRAP and for SPT was in the extent of labeling with the probe. For SPT it was necessary to use a low labeling of MHC class I so that the particles were well separated on the cell surface and thus could be unambiguously tracked from frame to frame. This level of labeling gave unacceptable noise levels in the FRAP experiments, and thus a higher labeling (achieved by using a higher probe concentration) was used to improve the signals. If not all MHC class I molecules have the same affinity for the probe, possibly as a result of clustering, then the labeling conditions might conceivably affect the results. We were able to perform a partial check on this supposition from the observation that the fluorescence of the illuminated spot in each FRAP recording varied considerably in brightness. The prebleach signal can be used as a measure of how heavily the illuminated area has been labeled, thus permitting a correlation to be sought between the extent of labeling and the diffusion coefficient and percentage recovery for individual FRAP curves. Fig. 4 shows that in fact no correlation was detectable.

A further possible explanation of the fast FRAP recoveries is that photobleaching of PhyE is rapidly reversible. We therefore performed a FRAP experiment with the probe immobilized on a polylysine-coated microscope slide. No fluorescence recovery occurred, demonstrating that reversible photobleaching is not a factor. In case there were other unforeseen properties of the PhyE-Fab probe that might account for the FRAP data, we performed additional FRAP experiments using a different probe consisting of OG514-labeled IgG. We found that the FRAP curves were similar to those obtained with PhyE. As shown in Table 1, the diffusion coefficients obtained by fitting the data by the standard model are close to those obtained with PhyE-Fab. This demonstrates that the larger PhyE-Fab probe does not experience steric hindrance to motion on the cell surface. The mobile fraction appears to be somewhat smaller for the OG-IgG probe, possibly because some cross-linking occurs with the divalent probe. This may also explain why the parameters failed to converge when attempts were made to fit the data by the anomalous diffusion model.

The SPT and FRAP measurements were performed on somewhat different time scales. SPT data were recorded over the time range 4–300 s with a maximum time resolution of 4 s between frames. FRAP data were recorded over a time range of 50 ms to 30 s. It is conceivable that the discrepancy in diffusion coefficients arises from the limited time resolution of the SPT measurements. We think this unlikely because the distances moved by MHC class I molecules (see Fig. 7 of Smith et al., 1999) are insufficient to account for the FRAP curves in the time range for which the two methods overlap. It is also conceivable that rapidly diffusing molecules are missed in the SPT experiment because the particle images are motionally blurred. We previously argued against this supposition (Smith et al., 1999) on the basis of a simulation that showed that an exposure time of 1 s would capture molecules having a diffusion coefficient of 10^{-9} cm^2 s^{-1}, whereas exposure times down to 0.3 s were employed for the SPT measurements. Nevertheless, in view of the FRAP data it is clearly important to carry out SPT with shorter exposure times and higher time resolution. We have recently modified our imaging system to permit these measurements to be performed.

Previous comparisons of FRAP and SPT measurements on cell membranes have been complicated by the fact that different probes are generally used for the two types of measurement. The different modes of motion often detected in an SPT experiment also mean that a comparison is not straightforward. Nevertheless, where comparisons have been made, the FRAP diffusion coefficients are often several-fold greater than the SPT values (Saxton and Jacobson, 1997). An exception is the diffusion of concanavalin A receptors on fish epidermal keratinocytes where both SPT and FRAP gave similar fast diffusion coefficients that surprisingly were little affected by receptor aggregation (Kucik et al., 1999). In one case, an anomalous diffusion model was used to interpret both SPT and FRAP measurements of $Fc_\epsilon RI$ on rat basophilic leukemia cells (Feder et al., 1996). SPT using fluorescent low-density lipoprotein conjugated to IgE as a probe gave a D (1 s) value of 9.6×10^{-11} cm^2 s^{-1}, some 30 times lower than the FRAP value obtained with fluorescent IgE of 3.0×10^{-9} cm^2 s^{-1}. Such discrepancies can conceivably be explained by the different nature of the probes used for FRAP and SPT, but this is not the case in the present study where the identical probe was used for both measurements.

The possibility that the intense photobleaching light used in FRAP experiments might damage the cell membrane has been extensively investigated. These experiments (summarized in Wolf et al., 1980) have not revealed any evidence for such damage. In particular, Wolf et al. (1980) found in a double-labeling experiment that extensive photobleaching of one fluorophore did not alter the FRAP data obtained with a second fluorophore. The slight possibility that photodamage is rapid and occurs to the same extent irrespective of the amount of photobleaching could not, however, be absolutely ruled out.

Although lower light intensities are used in SPT experiments, the samples could conceivably be photodamaged by the longer exposure required. We previously noted that this was unlikely to be a factor as there was no evidence for a progressive loss of mobility during an SPT experiment (Smith et al. 1999). As a further control in the current FRAP experiments, we subjected cells to a pre-illumination similar to that used for SPT. This had no detectable effect on the subsequent FRAP measurements.

In summary, both the FRAP and SPT data for MHC class I on HeLa cells are consistent with an anomalous diffusion model, and there is reasonable agreement for the value of the anomalous diffusion exponent. There is, however, a significant unexplained discrepancy in diffusion coefficients. In view of these findings, it will clearly be important to carry out further detailed comparisons of results from SPT and FRAP measurements using different cells and receptors.

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