Detection of Dimers of Dimers of Human Leukocyte Antigen (HLA)–DR on the Surface of Living Cells by Single-Particle Fluorescence Imaging

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Abstract. The technique of single-particle fluorescence imaging was used to investigate the oligomeric state of MHC class II molecules on the surface of living cells. Cells transfected with human leukocyte antigen (HLA)–DR A and B genes were labeled at saturation with a univalent probe consisting of Fab coupled to R-phycoerythrin. Analysis of the intensities of fluorescent spots on the cell surface revealed the presence of single and double particles consistent with the simultaneous presence of HLA-DR heterodimers and dimers of dimers. The proportion of double particles was lower at 37°C than at 22°C, suggesting that the heterodimers and dimers of dimers exist in a temperature-dependent equilibrium. These results are discussed in the context of a possible role for HLA-DR dimers of dimers in T cell receptor–MHC interactions. The technique is validated by demonstrating that fluorescence imaging can distinguish between dimers and tetramers of human erythrocyte spectrin deposited from solution onto a solid substrate. The methodology will have broad applicability to investigation of the oligomeric state of immunological and other membrane-bound receptors in living cells.

HUMAN major histocompatibility complex (MHC)[1] class II genes are expressed primarily on specialized antigen-presenting cells such as macrophages, B lymphocytes, and dendritic cells (for review see Trowsdale, 1994). The encoded products, known as human leukocyte antigen (HLA) class II, consist of a large array of noncovalently coupled transmembrane heterodimers of α and β glycoproteins grouped in at least three subsets designated HLA-DR, HLA-DQ, and HLA-DP. The most highly expressed among these subsets is HLA-DR, which is encoded by a nonpolymorphic A gene and up to five polymorphic B genes, depending on the haplotype. Class II molecules bind exogenously derived peptides in the endocytic pathway and present them at the cell surface where they act as a ligand for T cell receptors on $CD4^+$ cells.

A major advance in understanding the molecular basis of MHC class II function was the determination of a high resolution structure of the extracellular domain of HLA-DR1 by x-ray crystallography (Brown et al., 1993). Intriguingly, these molecules were found to crystallize as dimers of dimers (sometimes referred to as "superdimers"), prompting much speculation as to the possible existence and functional role of such entities in antigen-presenting cells (Ploegh and Beneroch, 1993; Germain, 1994; Fields and Mariuzza, 1996; Roucard et al., 1996).

In addition to MHC class II, there are a variety of cell surface receptors where the oligomeric state of the receptor is thought to be of functional significance. Evidence is accumulating that EGF and PGDF receptors, receptors of the tumor necrosis factor family, as well as membrane immunoglobulin all use dimerization as a key event in transmembrane signaling (Weiss and Littman, 1994; Heldin, 1995). There are also numerous experiments that suggest that oligomerization of T cell receptors is important in T cell activation (Sette et al. 1994; Takihama et al., 1994). It is thus highly desirable to develop methods for detecting, and preferably quantifying, the oligomeric state of cell surface receptors on living cells. Unfortunately, existing methods suffer from a number of limitations and drawbacks. For example, solubilization of cells by nonionic detergents followed by immunoprecipitation (Schäfer et al., 1994; Roucard et al., 1996) is an invasive technique in which the solubilization step is normally performed at low temperature, conditions under which artifactual associations may

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1. *Abbreviations used in this paper*: CLIP, class II–associated invariant peptide; Cy3.29-OSu, 5,5′-disulphato-1-(6-hexanoic acid *N*-hydroxysuccinimide ester)-1′-ethyl-3,3,3′,3′-tetramethylindocarbocyanine; HLA, human leukocyte antigen; LDL, low-density lipoprotein; MHC, major histocompatibility complex; PE, R-phycoerythrin; SMCC, succinimidyl *trans*-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; TCR, T cell receptor.

be induced. Fluorescence resonance energy transfer (Szollosi et al., 1989; Matko et al., 1994; Damjanovich et al., 1995; Jenei et al., 1997; Matko and Edidin, 1997) permits associations between receptors on living cells to be detected but gives little information on the extent or stoichiometry of such associations.

The method of fluorescent single-particle imaging, which was first introduced by Gross and Webb (1986), offers the possibility of performing a quantitative study of receptor oligomerization. This technique was originally applied to the investigation of clustering of low-density lipoprotein (LDL) receptors (Gross and Webb, 1986; Morrison et al., 1994) where a range of cluster sizes might be expected due to entrapment in coated pits. Fluorescent LDL particles were bound to cells and imaged using high-sensitivity digital fluorescence microscopy. Provided the receptors are saturated, the number of particles bound by a cluster of receptors is expected to equal the number of receptors in the cluster. The individual particles cannot be resolved by optical microscopy, but fluorescent spots corresponding to 1-, 2-, 3-...particles have intensities in the ratio 1:2:3.... Thus, the histogram of spot intensities provides information on the cluster-size distribution of the LDL receptors.

The above approach has hitherto not been used to determine the specific oligomeric state of a membrane protein. To achieve this goal, we have constructed a univalent probe consisting of a Fab fragment of a monoclonal antibody covalently bound to the phycobiliprotein, R-phycoerythrin (PE). Phycobiliproteins are light harvesting complexes from algae and cyanobacteria which have the advantages of small size (typically 11×6 nm) and uniform number of fluorophores per particle (Glazer, 1983). We have used this probe to determine the oligomeric state of HLA-DR molecules on the surface of living cells in an effort to gain an understanding of HLA–class II–mediated T cell activation.

For these experiments, we used human fibroblasts, designated M1DR1/Ii, which are transfected with genes coding for HLA-DR α and HLA-DR β chains and also the invariant chain, which assists in correct assembly of the class II heterodimer with exogenous peptide (Romagnoly et al., 1993). Whereas the untransfected fibroblasts are devoid of detectable class II expression, the transfected cells express fully assembled heterodimers at the cell surface and act as antigen-presenting cells (Lightstone et al., 1995). Thus these cells have the advantage of expressing a single subset of MHC class II (i.e., HLA-DR), eliminating possible cross-reactivity between specific monomorphic antibodies to HLA-DR with HLA-DQ or HLA-DP molecules. In addition, their large flat areas make them very suitable for particle imaging experiments. Preliminary imaging data obtained with these cells have previously been presented (Wilson et al., 1997).

To validate the technique, we have also demonstrated that fluorescence imaging can distinguish between dimers and tetramers of human erythrocyte spectrin deposited from solution onto a solid substrate. Like MHC class II molecules, spectrin consists of heterodimers of α and β chains. It exists in solution as a dimer-tetramer equilibrium (Ungewickel and Gratzer, 1978) and is thus a good model protein for investigating self-association using imaging.

Materials and Methods

Cells and Antibodies

The human transfectant fibroblast cell line, M1DR1/Ii, was kindly provided by Professor R. Lechler (Royal Postgraduate Medical School, Hammersmith Hospital, London, UK). They were cultured as previously described (Wilson et al., 1996; Smith et al., 1998). The cell line HB55 secreting L243, an antibody specific for monomorphic HLA-DR determinants, was obtained from the American Type Culture Collection (Rockville, MD). IgG and Fab fragments were purified from tissue culture supernatant or ascites fluid as previously described (Smith et al., 1997).

Labeling Fab with PE

Aliquots of purified Fab were labeled with the pyridyl disulfide derivative of PE obtained from Molecular Probes (Eugene, OR). In brief, 690 μg of Fab was dialyzed against 100 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 (phosphate buffer) and concentrated to 5 mg/ml in a Centristart-1 microconcentrator (Sartorius AG, Göttingen, Germany). Thiol-reactive maleimide residues were introduced into Fab by incubating with five molar equivalents of 5 mM succinimidyl *trans*-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) in DMSO for 2 h at room temperature. Excess SMCC was removed by extensive dialysis against phosphate buffer.

In parallel, 2.0 mg of PE, pyridyl disulfide derivative (average 1.9 pyridyl residues per molecule) were incubated with 50 mM DTT for 15 min at room temperature. Excess DTT was eliminated by dialysis against phosphate buffer. The PE was then incubated with the Fab for 20 h at 4°C in the dark. To prevent Fab disulfide reduction or aggregation, a 20 M excess of *N*-ethyl maleimide was then added.

Purification of PE-Fab

PE-Fab consisting of a 1:1 conjugate was purified from free PE, unlabeled Fab, and higher ratio conjugates on a Bio-Select 250-5 HPLC size exclusion chromatography column (Bio-Rad Laboratories, Hercules, CA). 300 μl of crude labeled Fab was injected onto the column at 0.10 ml/min, eluted with 50 mM Na_2HPO_4, 150 mM NaCl, pH 6.8, and then 200-μl fractions were collected. Integration was performed using ValueChrom® integration analysis software (Bio-Rad Laboratories). PE-Fab concentration was determined spectrophotometrically using a molar extinction coefficient of $1.96 \times 10^6\ M^{-1}cm^{-1}$ for PE.

Flow Cytometry

Nonspecific binding and the PE-Fab concentration required for saturation were determined by flow cytometry using trypsinized M1DR1/Ii cells. These were harvested by centrifugation at 80 *g* for 5 min at room temperature and washed three times with PBS supplemented with 0.02% BSA and 0.02% sodium azide (buffer A). PE-Fab was then diluted to the required concentration in buffer A and incubated with the cells for 30 min at room temperature. The cells were then washed three times with buffer A, once with PBS, and then examined for fluorescence using a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA), counting 10,000 cells/sample.

Spectrin Purification and Labeling

Spectrin was purified from human erythrocyte ghosts essentially as described (Ohanian and Gratzer, 1984). Spectrin tetramers were formed by incubating concentrated spectrin solutions at 29°C (Ungewickel and Gratzer, 1978). Tetramer formation was checked by nondenaturing PAGE. Dimeric or tetrameric spectrin was labeled with the probe 5,5′-disulphato-1-(6-hexanoic acid *N*-hydroxysuccinimide ester)-1′-ethyl-3,3,3′,3′-tetramethylindocarbocyanine (Cy3.29-OSu) from Biological Detection Systems (Pittsburgh, PA) essentially using the protocol provided with the probe. Protein was determined by the Bradford assay (Bradford, 1976) and Cy3.29-OSu by absorption spectroscopy using an extinction coefficient of $1.5 \times 10^5\ cm^2$/ mol at 552 nm. A labeling ratio of ~10 dye molecules/spectrin dimer was achieved by adding 10 μl of 1 M Na_2CO_3, pH 9.2, to 372 μg spectrin in 100 μl 5 mM sodium phosphate, pH 7.5, and then adding 36.7 μg Cy3.29-OSu dissolved in 125 μl distilled water and incubating for 30 min at either room temperature or 4°C. Unreacted probe was separated on a PD10 column (Pharmacia Biotech, Inc., Piscataway, NJ). Spectrin molecules (6 nM in 20 mM Hepes, 0.5 mM DTT, 0.1 mM EDTA, 150 mM

NaCl, pH 7.9) were bound to polylysine-coated slides as previously described (Morrison et al., 1994).

Fluorescence Digital Imaging Microscopy

For imaging experiments, PE-Fab was incubated with M1DR/Ii cells grown on 8-well Lab-Tek slides (GIBCO BRL, Gaithersburg, MD) for 30 min at 4°C at a concentration of 80 or 120 nM (the two concentrations gave indistinguishable results). They were washed three times in cold PBS, once in cold RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mg/ml sodium carbonate, and then covered and transferred to the temperature-controlled microscope stage for analysis. Some experiments were performed in which PE-Fab binding and imaging were performed at the same temperature; no difference from cells labeled at 4°C was detected. Nonspecific binding was further checked by incubating nontransfected (HLA-DR negative) cells with 80 nM PE-Fab; only ~10 particles were detected in an area corresponding to that typically used for analysis (data not shown). Samples with PE-Fab bound to polylysine-coated slides were prepared as previously described (Morrison et al., 1994).

Imaging was performed with an inverted microscope (Diaphot; Nikon Inc., Melville, NY) using a 50W mercury lamp and a 40× phase contrast objective. Wavelengths were selected by 525- and 575-nm filters in the excitation and emission beams and a dichroic mirror with cut-off at 545 nm (Omega Optical, Brattleboro, VT). A Peltier-cooled CCD camera (Wright Instruments, Enfield, Middlesex, UK) was attached to the video port of the microscope and the image focused onto an EEV CCD 02-06-1-206 back-illuminated detector. Image acquisition, storage, and display were performed using the AT1 image control software running on a 486 computer (Wright Instruments).

Image Analysis

Images of small fluorescent particles appear as diffraction-limited spots that cover a small number of the detector pixels, and whose profile is closely approximated by a Gaussian function. The spots were analyzed as previously described (Morrison et al., 1994). In brief, the pixel data in the vicinity of each individual spot was least-squares fitted by a two-dimensional Gaussian to obtain a value and standard deviation Z_j and σ_Z for the intensity of the j^{th} spot above local background. The Gaussian width and its standard error were used to select true diffraction-limited particle images; typically 150–200 spots within a focused area of the cell were analyzed, and <25% might be excluded as being too wide for reliable use in the intensity quantifications.

Fluorescent spot intensity distributions were created by two methods. Simple histograms with up to 50 bins were used in most instances for ease of interpretation but omitting the significance of the standard error σ_Z. Quasicontinuous distributions were also constructed by summing the spot contributions at a large number (512) of points in the fluorescence intensity axis; each contribution was a normal Gaussian, centered at Z_j, with width equal to σ_Z and area equal to one. Both types of distributions show broad overlapping peaks, as photon count deviations and variable photobleaching combine to increase the variance in the fluorescence signal.

The data from polylysine slide images were then fitted by a 1-particle log-normal distribution to obtain a mean 1-particle intensity Z_s and distribution width W_s. Cell intensity distributions were fitted by a model having additional terms for the first, second, etc. convolutions of this 1-particle distribution with itself, to represent the 2-particle, 3-particle, etc. clusters; associated with each term was a fractional contribution, e.g., P_1 the fraction of single particles. To allow for different photobleaching histories for the cell and control images, the model distribution in the cell images was allowed to vary from the control value by a photobleaching factor. In most cases, such control and cell data were fitted simultaneously by a common 1-particle function, using a global analysis technique (Morrison et al., 1994); this method was used for the spectrin dimer/tetramer data, as a control sample of pure dimer was not prepared.

Results

Oligomeric State of Spectrin

Spectrin was purified from erythrocyte ghosts and samples were prepared in which the protein was either predominantly dimeric or in which the equilibrium was significantly shifted towards the tetrameric state. Both samples were labeled with the fluorescent probe Cy3.29-OSu and deposited on a polylysine-coated slide for fluorescent imaging. It was found that individual spectrin dimers could be imaged with as few as six fluorescent probes per dimer. However, for quantitative measurements ~10 probes/dimer were required.

Fig. 1 *a* shows an example of the images that were obtained. Individual spots are the diffraction-limited images of individual spectrin dimers or higher oligomers. The intensities of the individual spots are determined and plotted as histograms (Fig. 1, *b* and *c*). The histograms are fitted to a series of peaks with fluorescence intensities in the ratio 1:2:3. In Fig. 1 *b* (spectrin mostly dimeric) >80% of the spots fall within the first peak. The width of the peak is probably mainly determined by the statistical variation in the number of probes per dimer and noise in the measurement. Fig. 1 *c* shows the corresponding result in a sample in which the equilibrium was shifted towards tetramers. The histogram now shows one peak in approximately the same position as in Fig. 1 *b* and a significant second peak at twice the intensity. This second peak is assigned to spectrin tetramers. In three separate determinations the mean number of spots contributing to the second peak was 40 ± 3% of the total (corresponding to 59 ± 2% of the total spectrin present).

PE- Fab

Probes for investigating the oligomeric state of HLA-DR were prepared by isolating Fab fragments of antibodies specific to HLA-DR and conjugating them in a 1:1 mol ratio to PE. PE-Fab will have a molecular mass higher than either of the two starting materials so that HPLC size exclusion chromatography will elute the PE-Fab first, followed by the free PE and Fab. Fig. 2 shows three major peaks corresponding to 1:1 PE-Fab (*1*), free PE (*2*), and unlabeled Fab (*3*). The solid bar indicates the 1:1 PE-Fab fractions collected for flow cytometry and imaging experiments.

The 1:1 PE-Fab was then tested for specificity and binding to the HLA-DR receptor on M1DR/Ii cells. FACS® analysis revealed a high fluorescence intensity when 80 nM PE-Fab was used (Fig. 3 *a*). However, when cells were incubated with PE-Fab in the presence of a 10-fold excess of unlabeled Fab (Fig. 3 *b*), then the peak fluorescence value was reduced by 87%, demonstrating that the PE-Fab had largely retained the binding specificity of the unlabeled Fab. Nonspecific binding of the PE-Fab was further checked by incubating cells with 80 nM unconjugated PE (Fig. 3 *c*), where the peak fluorescence value was reduced to 4% of that of PE-Fab. To test the concentration of probe required to label all the receptors to saturation, M1DR/Ii cells were incubated with different concentrations of PE-Fab, and analyzed by FACS®. It was found that the receptors were saturated by 80 nM PE-Fab (data not shown).

The PE-Fab particles were then imaged after binding them to polylysine-coated slides (Fig. 4 *a*). Histograms of the intensities of the individual particles are shown in Fig. 5. As expected, there is a single narrow peak demonstrating that the PE-Fab does not self-aggregate to any appreciable extent. This is particularly important, as self-aggregation would confuse the interpretation of experiments performed with

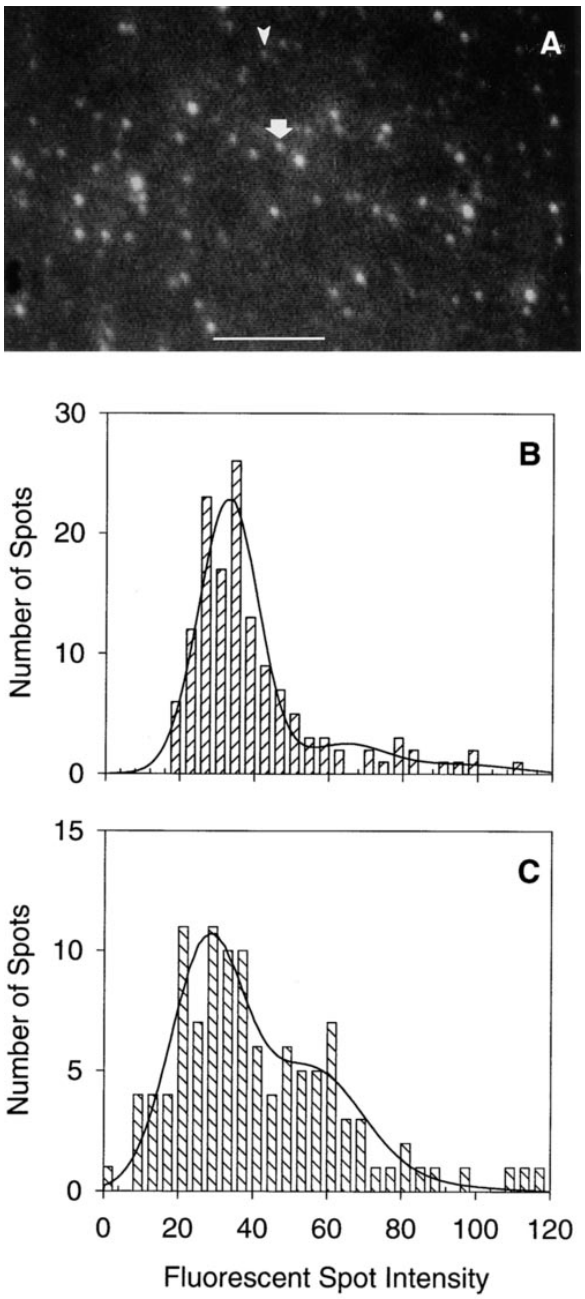

Figure 1. Analysis of dimer/tetramer composition of spectrin. (*A*) Image of fluorescent-labeled spectrin molecules (10:1 Cy3.29-OSu/spectrin dimer) bound to a polylysine-coated slide. The arrowhead identifies a spot of intensity corresponding to the maximum probability of the single-particle distribution; the broad arrow points to a spot having double this intensity. (*B*) Histogram of spot intensities for a predominantly dimeric sample. (*C*) Histogram of spot intensities for a sample incubated at 29°C to promote tetramer formation. Histograms are fitted to 1-particle, 2-particle, and 3-particle components as described in Materials and Methods. Bar, 10 μm (48 pixels).

these probes bound to cells. As the PE particles have a uniform number of fluorophores, the width of the peak arises largely from the noise in the measurement. The noise is a combination of the Poissonian distribution of the photon counts per pixel and electronic readout noise of the

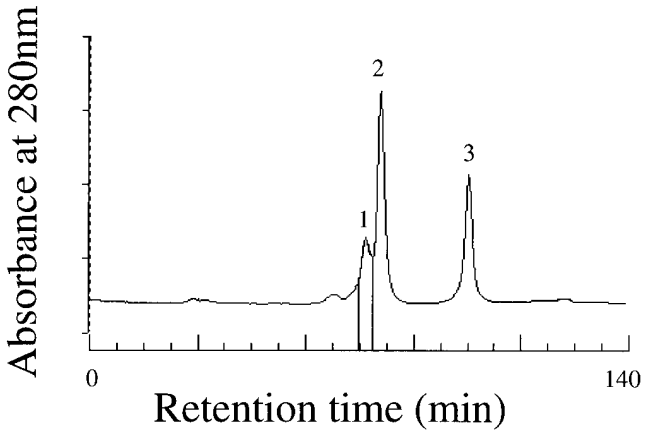

Figure 2. Purification of HLA-DR–specific PE-Fab by HPLC size exclusion chromatography. Labeled Fab fragments were loaded onto a Bio-Silect SEC 250-5 column (Bio-Rad Laboratories) and eluted with 50 mM Na_2HPO_4, 150 mM NaCl, pH 6.8, at 0.10 ml/min. Vertical lines indicate the fractions collected for further analysis.

CCD. Some photobleaching of the probe occurs during the measurement and also very probably contributes to the broadening of the intensity distribution.

Association of HLA-DR on M1DR/Ii Cells

The PE-Fab was then used to study the cell surface oligomeric state of HLA-DR receptors. Fig. 4 *b* shows part of an image of the PE-Fab bound to M1DR1/Ii cells. As well as dispersed particles, bright patches of fluorescence are sometimes seen, as at the left of Fig. 4 *b*. These patches can often be resolved into clusters of individual spots by using a different display contrast. For the analysis that follows, all spots in a given area of the cell are included which are of an appropriate width for a diffraction-limited image, irrespective of whether they are dispersed or form part of a cluster. Therefore, such spots correspond to individual receptors or aggregates of receptors with diameters smaller than that of the Airy disc (Inoue, 1989) (i.e., less than ~1 μm). Fluorescent patches that cannot be resolved into individual spots are not included in the analysis. Also excluded from the analysis of intensity histograms are spots that have widths greater than the diffraction limit. The integrated intensities of such spots suggest that they mainly arise from two particles that are sufficiently close together to be poorly resolved, but not close enough to display a diffraction-limited width. Very few of the excluded spots are bright enough to arise from higher order oligomers.

Examples of intensity histograms obtained at two different temperatures for PE-Fab bound to cells are shown in Fig. 5, *b* and *c*. It is evident that the images of particles bound to cells contain significant numbers of brighter spots compared with images of particles bound to polylysine-coated slides. The histograms in Fig. 5, *b* and *c* are fitted to obtain the proportions of spots corresponding to 1-, 2-, and 3-particles as described in Materials and Methods, and the parameters obtained are presented in Table I. An example of the quasicontinuous distribution described in Materials and Methods is given in Fig. 5 *i*; the values for the proportions of 1-particle spots etc. fell within the errors

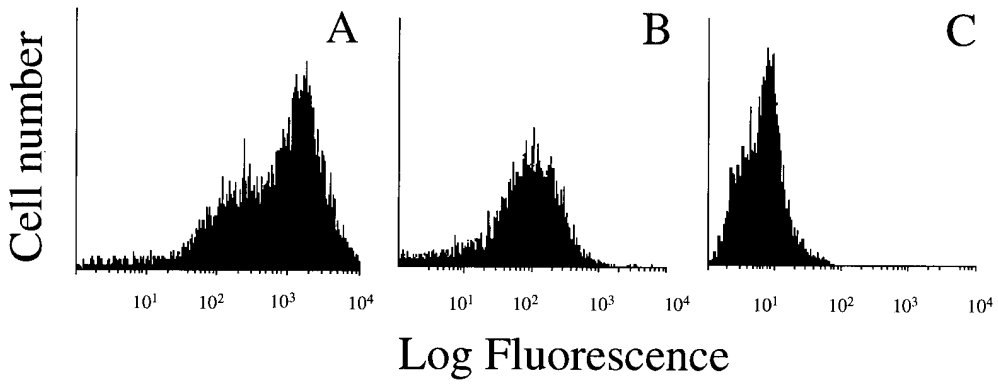

Figure 3. FACS® analysis of PE-Fab binding to HLA-DR receptors on M1DR/Ii cells. Cells were incubated with 80 nM PE-Fab in the absence (*A*) and the presence (*B*) of a 10-fold excess of the unlabeled Fab. Cells were also incubated with unconjugated PE (*C*). 10,000 labeled cells were analyzed for fluorescence on a FACScan® flow cytometer. The histograms display cell numbers (y-axis) as a function of relative fluorescence intensities (x-axis).

obtained for the parameters derived when fitting simple histograms, showing that the quasicontinuous distribution method was not a significant improvement.

Although these data are indicative of an association between HLA-DR molecules, there are other conceivable explanations for the difference between the spot intensities obtained with cells and polylysine-coated slides. The method of determining spot intensities, which subtracts the local background, should control for the possibility that errors arise from the additional and often variable background fluorescence that occurs with the on-cell measurements. However, as an additional control we performed imaging experiments in which the cells were labeled with a subsaturating concentration of PE-Fab. The results, presented in Fig. 5 *f* and Table I, show that the proportion of brighter spots decreases under these conditions, as one would expect if they correspond to multimeric receptors but not if they are the result of a measurement error. The fact that there is a reproducible difference in the histograms obtained at 22 and 37°C points to the same conclusion.

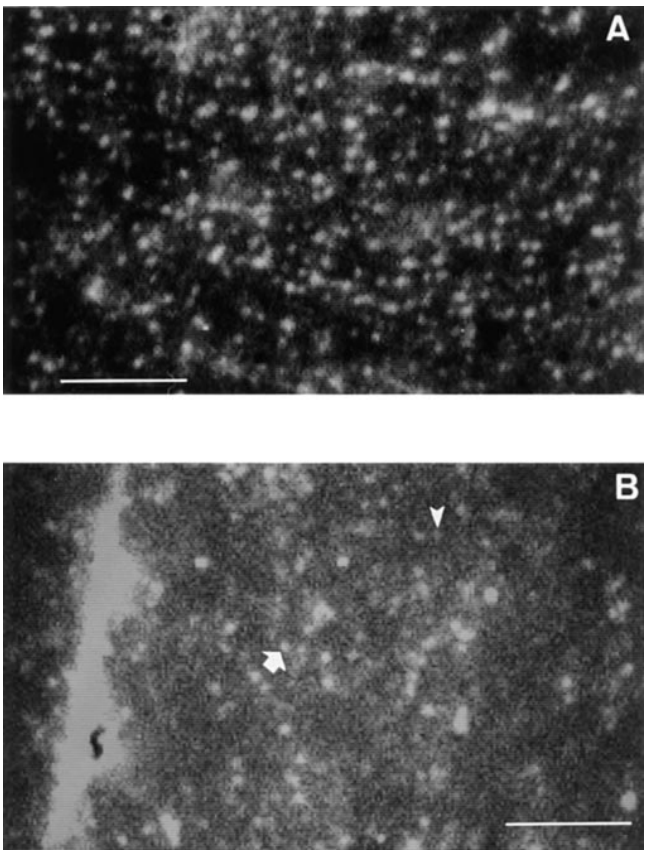

Figure 4. Images of PE-Fab. (*A*) Bound to polylysine-coated slide; (*B*) bound to M1DR1/Ii cell. The arrowhead and broad arrow identify high-probability 1-particle and 2-particle spots, respectively. The bright area towards the left of the image is on the edge of the cell. (It should be noted that the high contrast required to display the spots exaggerates differences in spot intensities and small variations in the background.) Bar, 10 μm (48 pixels).

A further potential problem is that a bright spot might result from two receptors that are not associated but are sufficiently close to one another to give a single diffraction-limited image (less than ~400 nm apart). This point was investigated by creating simulated images representing different surface concentrations of particles; all spots had diffraction-limited profiles with intensities equal to a value typical of PE-Fab, and were placed randomly and with synthetic noise equivalent to that measured with the CCD (Morrison, I.E.G., manuscript in preparation). These simulations were then analyzed by the techniques used for the cell images. The percentage of spots still deemed to represent single particles (P_1) was averaged over at least four simulations at each density and is given in Table II. At the spot densities found in cell images (0.1–0.24 spots/μm^2), the data suggest that no more than 7% of the spots would appear as false doubles by this mechanism.

Even if the spot intensities correctly reflect receptor associations, it is possible that the associations are influenced by labeling with the PE-Fab. There is no evidence that that the PE-Fab self-associates in solution or when bound to polylysine-coated slides, but it is conceivable that Fab, which can have a tendency to aggregate in solution, might mediate associations after collisions of mobile receptors. Therefore, we performed an experiment in which cells were labeled with PE-Fab at 4°C (where receptor mobility is negligible) and warmed to 22°C in the presence of excess free Fab before measurement. No significant differences in the intensity histograms were detected in this experiment (Fig. 5 *e* and Table I), thus ruling out the possibility of PE-Fab–mediated associations.

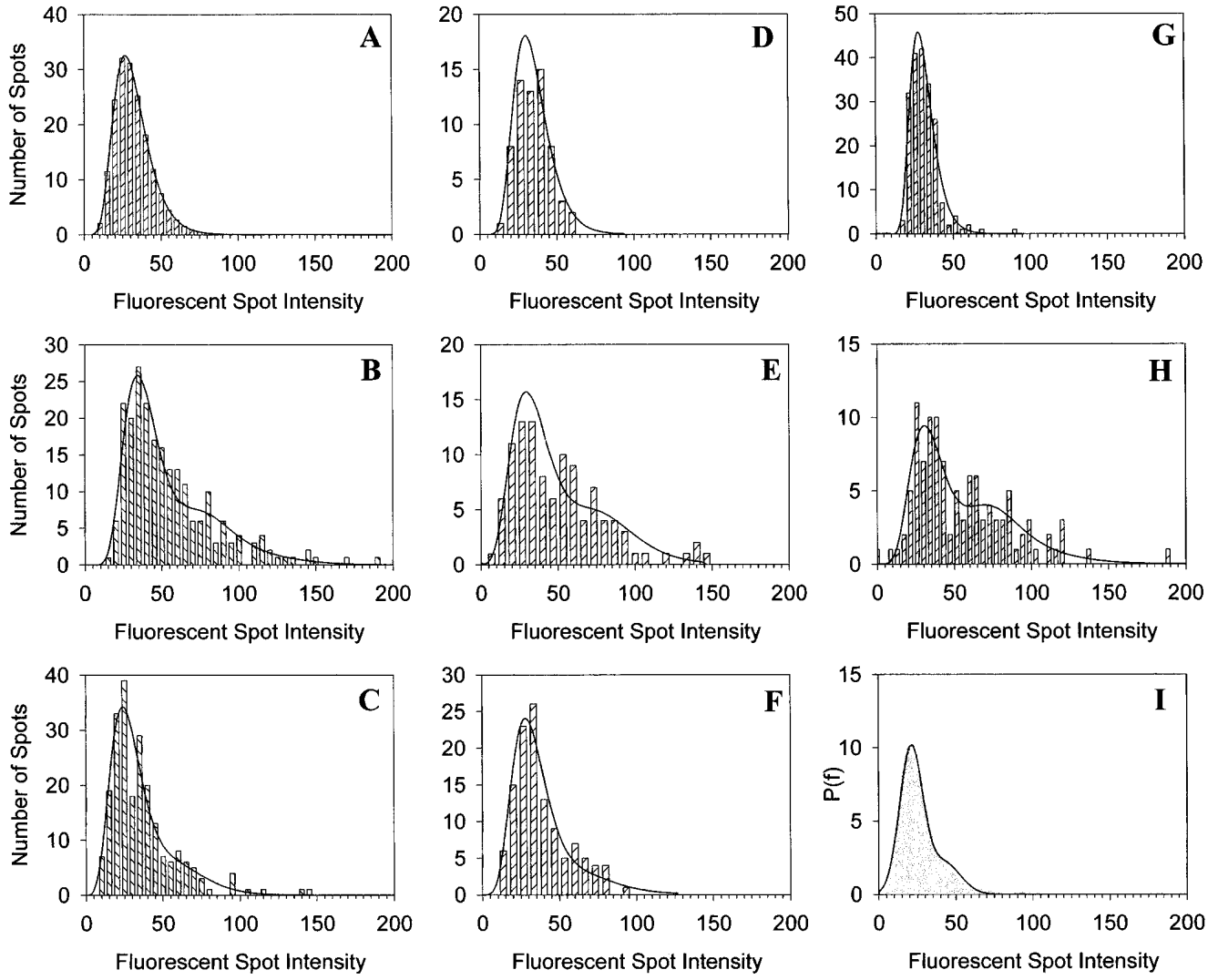

Figure 5. Histograms of spot intensities obtained from images of PE-Fab. (*A*, *D*, and *G*) Bound to a polylysine-coated slide; *A* is control for *B* and *C*, *D* is control for *E* and *F*, *G* is control for *H*. (*B*) Bound to M1DR1/Ii cells and imaged at 22°C. (*C*) Bound to M1DR1/Ii cells and imaged at 37°C. (*E*) As *B* but a 10 times excess of unlabeled Fab was added to the cells at 4°C before imaging at 22°C. (*F*) As *B* but cells were labeled with a subsaturating concentration of PE-Fab (40 nM). (*H*) As *B* but cells were incubated with 60 μM CLIP for 48 h at 37°C before imaging. (*I*) As *C* but the spot data have been formed into a quasicontinuous distribution rather than a histogram, and *P*(*f*) represents the relative fluorescence probability. (*A, D, G*) are fitted to a log-normal distribution, (*B, C, E, F, H,* and *I*) are fitted to 1-particle, 2-particle, and 3-particle components as described in Materials and Methods.

It is conceivable that HLA-DR molecules with an empty peptide-binding groove could bind to one another or to another membrane protein (Stern and Wiley, 1992). However, it has been shown that the peptide-binding groove is largely occupied by the class II–associated invariant chain peptide (CLIP) in the M1DR/Ii cells used in the present experiments (Lechler et al., 1996). However, to ensure saturation of the peptide-binding groove, cells were pulsed with CLIP peptide before imaging with PE-Fab at 22°C. This procedure had no significant effect on the proportions of 1-particle and 2-particle spots determined from the intensity histograms (Fig. 5 *h* and Table I).

Discussion

Single-particle fluorescence imaging has previously been used to study clustering of LDL receptors (Gross and Webb, 1986; Morrison et al., 1994) but not to determine the oligomeric state of a membrane protein. To demonstrate that the method can distinguish different oligomeric states, we performed measurements with a simple model system consisting of spectrin dimers and tetramers bound to a microscope slide. The spectrin dimers were labeled at a ratio of 10 fluorophores per dimer. Although imaging is possible with lower ratios (imaging single fluorophores is feasible; Sase et al., 1995; Schmidt et al., 1996), the Poissonian distribution in the number of probes per dimer results in unfavorably broad intensity distributions at the lower labeling ratios. Fig. 1 demonstrates that the intensity histograms exhibit two peaks whose magnitudes vary as expected according to the oligomeric state of the spectrin preparation. The conversion of dimers to tetramers deduced from

Table I. Analysis of Spot Intensity Histograms for PE-Fab Bound to M1DR1/Ii Cells

°C	*n*	P_1 (%)	P_2 (%)	P_3 (%)	Percentage of heterodimers	Percentage of dimers of dimers
22	9	66 ± 7	30 ± 3	4 ± 2	48 ± 5	43 ± 5
37	8	82 ± 6	15 ± 5	3 ± 3	68 ± 5	25 ± 8
22*	3	69 ± 4	30 ± 3	1 ± 1	52 ± 3	45 ± 5
22‡	4	96 ± 5	3 ± 5	1 ± 1	91 ± 5	6 ± 9
22§	3	63 ± 10	35 ± 9	2 ± 2	45 ± 7	50 ± 6

Histograms of fluorescent spot intensities are analyzed into 1-particle (P_1), 2-particle (P_2), and 3-particle (P_3) components as illustrated in Fig 5. *n* is the number of independent determinations, each on a different cell. Percentage of HLA-DR existing as heterodimers is calculated as $100P_1/(P_1 + 2P_2 + 3P_3)$, and percentage of HLA-DR–forming dimers of dimers as $200P_2/(P_1 + 2P_2 + 3P_3)$.
*Cells imaged in presence of excess unlabeled Fab (Fig. 5 *E*).
‡Cells labeled with a subsaturating concentration of PE-Fab (Fig. 5 *F*).
§Cells fed with CLIP (Fig. 5 *H*).

Fig. 1 *c* is less than previously described (Ungewickel and Gratzer, 1978) because some reconversion to dimer occurred during labeling.

Having established that the imaging technique can distinguish spectrin dimers and tetramers, the approach was used to investigate the oligomeric state of HLA-DR on living cells. We found that the images of PE-Fab particles bound to HLA-DR on M1DR1/Ii cells contain a significant proportion of bright spots that are not observed for single particles bound to polylysine-coated slides. We conclude that the on-cell spots arise almost entirely from single and double particles which in turn correspond to 1-molecule and 2-molecules of HLA-DR. The density of these molecules at the cell surface is such that there is a low probability of two HLA-DR molecules coincidentally being closer than the resolution of the microscope and thus these two molecules form an association. The absence of a significant number of higher order spots is strongly suggestive of a specific association which, in view of the crystallographic evidence (Brown et al., 1993), can reasonably be supposed to be the dimer of dimers.

The analysis could be in error if steric hindrance prevents two probes binding to a HLA-DR dimer of dimers. This, however, seems unlikely. The epitope to L243 has been previously mapped to residues on both α and β chains close to the peptide-binding groove (Fu and Karr, 1994). The hydrophilic domain of a class II heterodimer is ~8 × 7 nm (Brown et al., 1993), Fab is 8 × 5 nm (Polzak et al., 1973), and phycoerythrin is 11 × 6 nm (Ficner et al., 1992). Based on these dimensions, a model of the receptor with bound probes (Cherry et al., 1997) indicates that there is ample space for two particles to bind simultaneously to a dimer of dimers. Even if steric hindrance did occur, the data would still provide evidence for an associated state of the HLA-DR molecules.

The analysis in Fig. 5 and Table I applies to all spots that have a diffraction-limited width, i.e., they are images of an object that has a diameter of less than ~1 μm. This would include not only single and double particles but also much higher order clusters if they existed. In addition to such diffraction-limited spots, cells often exhibit patches of fluorescence of diameter 1 μm or more in which individual spots are unresolved. These patches most likely correspond to a high local density of HLA-DR molecules although the mechanism by which they are concentrated into a patch is unclear.

It is noteworthy that the proportion of dimers of dimers on the transfected cells is lower at 37°C than at 22°C, suggesting that they are in a temperature-dependent equilibrium with single heterodimers. It is reasonable to suppose that such an equilibrium is a property of HLA-DR in a lipid environment and thus occurs in all antigen-presenting cells. The proportion of molecules existing as dimers of dimers would be expected to depend on the concentration of HLA-DR in the membrane and possibly on the lipid composition.

The existence of MHC class II dimers of dimers on cell surfaces has previously been suggested by immunoprecipitation from detergent lysates (Schäfer et al., 1994; Roucard et al., 1996). The problem with this approach is that it is uncertain to what extent weak associations are preserved and artefactual associations are induced by the extraction procedures used. Previous biophysical approaches to studying receptor associations include fluorescence resonance energy transfer (Szollosi et al., 1989; Matko et al., 1994; Damjanovich et al., 1995) and luminescence quenching (Matko et al., 1995) measurements using flow cytometry. However, such measurements cannot distinguish and quantify different sized oligomers or larger aggregates. Correlation analysis of images obtained from confocal microscopy is an alternative method with the potential to answer such questions (Petersen et al., 1993; Huang and Thompson 1996); but the fluorescent beads used in these studies are much larger and more photostable than PE, and application of this technique to such low-intensity images as those obtained with PE has not been attempted. The single-particle imaging technique used here thus represents a significant advance in the study of MHC associations in cell membranes.

The possible functional significance of MHC class II dimers of dimers has been much discussed (Brown et al., 1993; Ploegh and Beneroch, 1993; Germain, 1994; Schäfer et al., 1994; König et al., 1995; Sakihama et al., 1995; Fields and Mariuzza, 1996; Garban et al., 1996; Roucard et al., 1996). The dimer of dimers could strengthen interactions with T cells by simultaneously binding two T cell receptors (TCR) (Brown et al., 1993; Fields and Mariuzza, 1996).

Table II. Analysis of Spot Intensity Histograms from Simulated Images of Uniform Particles at Increasing Surface Concentrations

	Mean ± SD			
Mean inter-spot distance (μm)	1.66 ± 0.06	1.23 ± 0.05	1.07 ± 0.03	0.99 ± 0.03
Spot surface density (μm^{-2})	0.129 ± 0.005	0.228 ± 0.006	0.32 ± 0.01	0.41 ± 0.02
Single particle fraction P_1 (%)	98.2 ± 1.2	94.9 ± 1.6	92.7 ± 3.4	90.2 ± 1.3

Particles having uniform fluorescence intensities and diffraction limited profiles were placed at random positions on a pixel grid with synthetic noise. For each surface concentration, at least four simulated images were analyzed; at each spot, the nearest neighbor was selected, the distances were averaged, and the mean surface density calculated. The image analysis used for the PE-Fab on cells was then applied to obtain the fraction of single-particle spots, giving a mean and standard deviation at each surface density.

Association of TCRs could constitute a signaling mechanism, as is the case for various cell surface receptors (Weiss and Littman, 1994; Heldin, 1995). A problem with such hypotheses is that the two MHC heterodimers are unlikely to be loaded with the same peptide (Harding and Unanue, 1990), although the probability would be increased in infected cells where many of the presented peptides would be derived from the same antigen. More dynamic models have also been proposed involving exchange of MHC molecules between dimers of dimers or weak TCR associations that are stabilized (and hence activated) only when binding a homogeneous peptide–MHC dimer of dimers (Fields and Mariuzza, 1996). Reich et al. (1997) have shown very recently that the extramembraneous domains of TCR and MHC class II molecules can oligimerize in solution to form supramolecular structures.

This study provides strong evidence for the existence of dimers of dimers in a membrane environment. On the other hand, the proportion of dimers of dimers is quite low at physiological temperature, indicating that they do not have a high association constant. The transfected cells used in these present studies do, however, have a relatively low surface density of HLA-DR; it is possible that in other antigen-presenting cells there is a greater degree of association resulting from a higher surface density of these molecules. It is also possible that patches of more concentrated receptors, as observed in this study, could provide a favorable site both for dimer of dimers formation and for T cell interaction. It should be noted that these patches are too large to correspond to the hexamers of MHC class II recently observed in solution studies by Reich et al. (1997). Moreover, these higher oligomeric states were only detected in the presence of the extramembraneous domain of the TCR that formed supramolecular complexes with MHC class II–peptide complexes.

In conclusion, single-particle imaging provides a method of detecting and determining the extent of dimer of dimers formation of MHC class II molecules in living cells. Thus, it should now be possible to determine the significance of these entities by combining this approach with functional studies of mutated HLA-DR molecules. The methodology should also be readily applicable to variety of immunological and other receptors whose association state is likely to be relevant to their functional properties.

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