Rotational and lateral dynamics of I-A<sup>k</sup> molecules expressing cytoplasmic truncations

Heidi M. Munnelly, Cynthia J. Brady, Guy M. Hagen, William F. Wade<sup>2</sup>, Deborah A. Roess<sup>1</sup> and B. George Barisas

Departments of Chemistry and <sup>1</sup>Physiology, Colorado State University, Fort Collins, CO 80523, USA
<sup>2</sup>Department of Microbiology, Dartmouth Medical School, Lebanon, NH 03756, USA

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Abstract

Rotational and lateral diffusion of I-A<sup>k</sup> molecules with various α and β chain cytoplasmic truncations known to affect class II function were measured to assess the role of cytoplasmic domains in regulating I-A<sup>k</sup> molecular motions. Deletion of all 12 α chain C-terminal residues and all 18 corresponding β chain residues (α<sub>12</sub>/β<sub>18</sub>) is known to abrogate translocation of protein kinase C to the nucleus upon class II cross-linking. Similarly, truncation of the entire cytoplasmic α chain domain and the 10 C-terminal residues of the β chain impairs presentation of antigenic peptides to T cells. The rotational correlation time of the wild-type molecule, 11.9 ± 2.6 µs as measured by time-resolved phosphorescence anisotropy, decreased to 7.2 ± 3.7 µs in the fully truncated α<sub>12</sub>/β<sub>18</sub> protein. Other truncated class II molecules exhibited only small changes in molecular rotation rates relative to the wild-type. The rate of lateral diffusion of the fully truncated molecule, measured with two independent methods, 2.3 × 10<sup>-10</sup> cm<sup>2</sup>/s, was comparable with that of the wild-type molecule. Thus, it appears that the α and β chain cytoplasmic domains regulate the molecular motions of unperturbed I-A<sup>k</sup> molecules only modestly, despite the known involvement of these regions in class II signaling. Various explanations for this behavior are discussed, e.g. the possibility that class II membrane complexes are sufficiently large that association and dissociation of specific signaling proteins during antigen presentation do not significantly perturb the apparent molecular motions of the complex.

Introduction

Class II molecules of the MHC are central to antigen presentation in T cell-dependent immune responses. MHC class II molecules are responsible for intercellular recognition and self/non-self discrimination, and these molecules thus play a large role in tissue and organ rejection. MHC class II molecules are αβ heterodimers which are expressed on the surfaces of antigen-presenting cells, such as macrophages, B lymphocytes and dendritic cells. These molecules present antigenic peptides to T cells during immunological responses and they are, therefore, critical molecules in the function of the immune system (1). The 34–36 kDa α chain contains 12 amino acid residues in the cytoplasmic tail, while the 27–29 kDa β chain contains 18 cytoplasmic residues.

Structural modifications of MHC class II molecules allow assignment of function to specific molecular domains, i.e. protein kinase C (PKC) translocation and antigen presentation (2). Several transfected M12.C3 B cell lymphomas expressing murine I-A<sup>k</sup> MHC class II molecules with cytoplasmic truncations of the α and β chains have been developed (3). Deletions of the six membrane proximal cytoplasmic amino acid residues of the β chain abrogates the translocation of PKC from the cytoplasm to the nucleus upon class II molecule cross-linking (3). As compared to the wild-type molecules, class II molecules with fully truncated α chains paired with partially truncated β chains where the six membrane proximal residues remain, α<sub>12</sub>/β<sub>12</sub>, showed altered but substantial PKC signaling. (A typical abbreviation for a truncated class II molecule would be ‘α<sub>12</sub>/β<sub>12</sub>’ indicating truncation of the 12 C-terminal α chain residues paired with a wild-type β chain.) In contrast, the fully-truncated
molecules with the phenotype α-12/β-18, where 12 amino acids and 18 amino acids were deleted from the α and β chains respectively, is not able to translocate PKC from the cytoplasm to the nucleus. This might suggest that the cytoplasmic regions of class II are physically coupled to proteins that mediate signal transduction (3). The 12 cytoplasmic residues of the α chain and the 10 membrane proximal residues of the β chain have been implicated in antigen presentation since this function is impaired when these residues are deleted (4–6). Again, this suggests that deletions of the specific cytoplasmic sites may alter the ability of class II molecules to participate in intermolecular interactions of functional significance.

Measurements of membrane dynamics of class II molecules provide a window on such functional interactions with other membrane species. Rates of molecular rotation can be quantitated through the rotational correlation time, a quantity measured by time-resolved phosphorescence anisotropy. The rotational correlation time of a protein linearly reflects the size of the functional complex with which it moves (7). Therefore, this quantity is particularly useful in measuring molecular interactions such as might exist between the cytoplasmic tails of I-AK and associated signaling proteins. Since the α and β chains of class II interact in some manner with the cytoskeleton (8) or with signaling proteins, the rates of rotational and lateral diffusion of these molecules might increase as amino acids are deleted from the cytoplasmic tails of these class II molecules. The rotational diffusion of wild-type MHC class II molecules showing enhanced antigen presentation following paraformaldehyde fixation has been measured previously using time-resolved phosphorescence anisotropy (9). Time-resolved phosphorescence anisotropy measurements performed on MHC class II molecules possessing α and β chain truncations might be expected to provide additional insight into the molecular organization of class II molecules on the plasma membrane.

Truncations of cytoplasmic tails might also be expected to increase the rates of lateral motion. Indeed, previous spot fluorescence photobleaching recovery (FPR) experiments performed on the I-AK cell lines expressing MHC class II molecules with truncated cytoplasmic tails suggested that, as the number of amino acids deleted from the cytoplasmic domain increased, the rate of I-AK lateral diffusion also increased (10). The measurement of lateral diffusional parameters has become more sensitive and reproducible with the recent development of interferometric fringe FPR (11). This method can increase fluorescence signal levels as much as 100-fold over conventional spot methods. Mutant proteins on transfected cells are frequently expressed at low levels on the plasma membrane (3). Therefore, spot FPR measurements on such cells are complicated by the low fluorescence signals and associated poor signal-to-noise ratios. With the recent refinement of interferometric fringe FPR, it is now possible to re-examine such transfected cells expressing cytoplasmically truncated MHC class II molecules with the expectation of greatly improved results.

Another possible relation between class II interactions and molecular dynamics concerns the signaling pathways of K46J and M12.C3 cell lines. At 2–4 min after extensive cross-linking I-AK, the K46J line exhibits activation of Ca^{2+} flux and the activation of protein tyrosine kinase (12). This signaling phenotype of MHC class II is expressed on I-AK-positive B cell lymphomas which have been transfected with α-12/β-18 I-AK truncates as described above. As amino acids are deleted from the α and β chains, this alternative signaling pathway is not affected (12). This result suggests that interactions between class II and other proteins exist in the K46J cell lines that do not exist in the M12.C3 cell lines, and it might therefore be reflected in general differences in the molecular motions of I-AK between these two lines.

Finally, molecular motions of MHC class II in the plasma membrane are directly important to immunological function of these molecules. Lateral motion of class II molecules is intrinsic to the molecules’ steady-state trafficking (13). Moreover, movement of these molecules into the ‘immunologic synapse’ where they interact with TCR (14) involves changes in their membrane localization and organization (15). Last, recent reports show that class II is resident either in, or closely associated with, plasma membrane rafts (16), suggesting that class II signaling requires the molecular capacity to localize properly within the membrane.

Methods

Chemical reagents

L-Glutamine, sodium pyruvate, 2-mercaptoethanol, glucose, glucose oxidase and catalase were purchased from Sigma (St Louis, MO). FBS was purchased from Summit Biotechnologies (Fort Collins, CO). RPMI 1640 and penicillin/streptomycin were purchased from Irvine Scientific (Santa Ana, CA). Geneticin (G-418) was purchased from Gibco (Grand Island, NY). Tetramethylrhodamine isothiocyanate (TRITC), FITC, erythrosin isothiocyanate (ERITC) and [6-(tetramethylrhodamine-5(and 6)-carboxamido)hexanoic acid succinimidyl ester (TAMRA) were purchased from Molecular Probes (Eugene, OR), M12.C3 and K46J cells expressing wild-type I-AK and various cytoplasmic amino acid residues truncations were prepared as described previously (10,12).

39J Fab preparation and fluorescent probe derivatization

Spent tissue culture supernatant was passed through a Protein A column and 39J was recovered in the pH 2.5 eluate which was neutralized and dialyzed against PBS. 39J Fab fragments were prepared using the ImmunoPure Fab preparation kit (cat. no. 44885) produced by Pierce (Rockford, IL). Briefly, use of this kit involved the digestion of 39J anti-α chain mAb with a 50% glycerol and water slurry of 250 µg/ml papain immobilized on an agarose support, pH 10. After a 5 h incubation at 37°C, Fab fragments were decanted from the slurry and isolated by passing the crude digest over a Protein A column. The Fab fragments washed through with the binding buffer, pH 7. The successful preparation of the 50 kDa 39J Fab fragments was confirmed by SDS–PAGE using a 10–20% Tris–glycine gel under reducing conditions.

The TRITC–39J antibody conjugate and the ERITC- and TAMRA-derivatized 39J Fab fragments were prepared using standard methods (17). The FITC-derivatized 39J Fab fragments were prepared using a modification of the method described by Mishell and Shiigi (18). The dye-to-
protein ratios of the conjugates were 2.0 mol TRITC/mol intact 39J mAb, 0.8 mol TAMRA/mol 39J Fab, 0.8 mol ErITC/mol 39JFab and 0.4 mol FITC/mol 39J Fab. Prior to use, all phosphor- orfluorophore-derivatized proteins were centrifuged at 130,000 g for 10 min in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA) to remove any protein aggregates which might have formed during storage at 4°C. These cells expressing I-A<sup>k</sup> molecules have been shown previously to be particularly sensitive to aggregation of antibody labels during storage since preliminary rotational measurements, where probes were not adequately airfuged before labeling, showed rising anisotropies now know to be artifactual (19).

**Cell preparation and labeling for time-resolved phosphorescence anisotropy and FPR measurements**

M12.C3 cells expressing wild-type I-A<sup>k</sup> and cytoplasmic amino acid residue truncations of I-A<sup>k</sup> were grown in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 × 10<sup>–5</sup> M 2-mercaptoethanol, 300 µg/ml G-418 and 1 mM sodium pyruvate. The K46J cells expressing wild-type I-A<sup>k</sup> and cytoplasmic amino acid truncations of I-A<sup>k</sup> were grown in the same medium described above except 1 mg/ml G-418 was added. For the phosphorescence anisotropy measurements, 10<sup>7</sup> cells in 250 µl were labeled with 2.0 µM ErITC-derivatized 39J Fab fragments for 30 min on ice. After labeling, cells were washed 2 times by centrifugation at 300 g for 3 min in BSS to remove any unbound probe. Prior to the time-resolved phosphorescence anisotropy experiments, cells were treated with 50 mM D-glucose, 0.2 mg/ml glucose oxidase (Sigma type II-S) and 0.25 mg/ml catalase (Sigma type C-10) for 15 min to eliminate triplet quenching by O<sub>2</sub> (20). For the FPR measurements, 10<sup>6</sup> cells in 50 µl were treated with 2.0 µM TRITC-, FITC- or TAMRA-derivatized 39J mAb or 39J Fab fragments for 30 min on ice. Cells were washed once with BSS, centrifuged for 3 min at 300 g and resuspended in 0.5 ml BSS prior to the FPR experiments.

**Time-resolved phosphorescence anisotropy instrumentation and analysis**

Time-resolved phosphorescence anisotropy experiments were performed as previously described (9, 21). The time-resolved phosphorescence anisotropy apparatus is shown Fig. 1. Washed and deoxygenated cells were placed in a 5 mm Suprasil quartz cuvette mounted in a thermostated housing, typically maintained at 4°C unless otherwise specified. Excitation was via the frequency-doubled 532 nm output of a Spectra-Physics DCR-11 Nd:YAG laser operated at 10 Hz with a vertically polarized TEM <sub>00</sub> output of 250 µJ at the sample cuvette. Sample phosphorescence was isolated by a 1 M Na<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> solution chemical filter, a KV 550 color filter (Schott Glass Technologies, Duryea, PA) to block scattered light, a 3 mm thick RG 665 filter to eliminate delayed fluorescence, and a rotating polarizer to select phosphorescence emitted with vertical I<sub>||</sub>(t) and horizontal I<sub>\perp</sub>(t) polarizations. The phosphorescence signal was detected by a thermoelectrically cooled EMI 9816A photomultiplier tube protected from light scattered during laser pulses by a fast gating circuit (22). The photomultiplier output was amplified by a Tektronix 476 oscilloscope and a 35 MHz bandwidth buffer amplifier, and 4096 traces in each polarizer orientation averaged by a Nicolet 12/70 computer equipped with a 20 MHz, 8-bit ADC. Data were downloaded into an Intel 80386 IBM-PC-compatible computer for analysis. Phosphorescence intensities I<sub>||</sub>(t) and I<sub>\perp</sub>(t) were analyzed (23) to yield a phosphorescence intensity function s(t) = I<sub>||</sub>(t) + 2I<sub>\perp</sub>(t) and a phosphorescence anisotropy function r(t) = (I<sub>||</sub>(t) − I<sub>\perp</sub>(t))/s(t). The phosphorescence intensity function was fitted to a sum of two exponential decays plus a constant term, i.e. an arbitrarily slow decay. To facilitate comparison of phosphorescence decay between samples, mean decay half-times were calculated from the lifetimes and amplitudes of each step. Results from the lifetime analysis were used to weight points in a non-linear least squares fit of the anisotropy data. The anisotropy decay for an uniaxial rotator labeled with randomly oriented chromophores should exhibit two rotational correlation times but cellular data are rarely precise enough to permit resolution of the exponentials. Anisotropy data were thus analyzed according to a single average exponential decay model which provided satisfactory fits to all our decay curves. In these analyses the weight of a given anisotropy point was set proportional to the intensity calculated at the corresponding time:

\[
r(t) = r_0 + (r_\infty - r_0) \exp(-t/\tau)
\]

Fitting r(t) to equation (1) yields the initial anisotropy value r<sub>0</sub>, the limiting anisotropy value r<sub>\infty</sub> and the rotational correlation time \( \tau \) as well as the statistical uncertainties in these quantities (24). The initial and final anisotropies are measures of restrictions to protein rotational motion, while the rotational correlation time is proportional to the in-membrane volume of the rotating species. It is on this later quantity that we have focused our attention in this study.

**FPR instrumentation and analysis**

Equipment and methods for FPR measurements have been published elsewhere in detail (11). The microscope photometer is based on the Zeiss Axioim microscope equipped with a Zeiss x63, NA 1.00, Plan Neofluor immersion fluorescence objective, a fluorescence vertical illuminator and an MP03 photometer module. Standard Zeiss barrier and exciter filters were used, but Omega dichroic mirrors 510DRLPO2 and 540DRLPO2 were essential for fringe measurements involving fluorescein and tetramethylrhodamine respectively. Cells were examined under cover slip on well slides on a thermoelectrically cooled/heated stage. Measurements were typically performed at 23°C, although temperatures of 4–37°C were occasionally employed.

A Coherent Radiation Innova 100 argon ion laser provided excitation at 488 nm for fluorescein-derivatized proteins and at 514 nm for rhodamine-derivatized proteins. For spot measurements, the laser spot had a 1/e<sup>2</sup> radius of 0.41 µm. The power during the bleaching pulse for the spot method was 9 mW and, in the probe beam, 3 µW. In fringe measurements the illuminated region had a 1/e<sup>2</sup> radius of 17 µm with laser powers of 1.6 W in the bleaching pulse and 500 µW in the probe beam. The fringe method interferometer divided the laser beam into two equal intensity components separated by a center-to-center distance of 5.5 mm (11) giving a fringe spacing at the sample of...
Rotational and lateral motions of truncated I-A<sup>k</sup>

Fig. 1. Apparatus for time-resolved phosphorescence anisotropy measurements of cell surface protein rotational diffusion. The functions of the components are described in the text.

1.75 µm. Typical experiment run times were 15 s prebleach and 25 s postbleach with 50 ms/point data acquisition. Fringe and spot FPR bleaching times were 800 and 100 ms respectively.

The equations defining fluorescence recovery kinetics for spot photobleaching and for fringe photobleaching of spherical samples have already been presented elsewhere in detail (11). Unadjusted raw data were represented directly in terms of the various parameters associated with a given measurement including the prebleach and immediate postbleach fluorescence levels <i>F</i><sub>0</sub> and <i>F</i><sub>−</sub>, the percentage of fluorophores %M mobile on the timescale of the experiment, and an appropriate function representing the recovery kinetics in terms of a decay half-time <i>t</i><sub>1/2</sub>. The parameters were evaluated directly by Marquardt non-linear least-squares procedures (25).

From the measured time at which fluorescence recovery is half complete and from the known optical parameters, the desired diffusion constant could then be evaluated.

Results

Phosphorescence of erythrosin-39J Fab bound to cell surface I-A<sup>k</sup> exhibits a three-component decay.

Representative decay traces for the phosphorescence intensity of erythrosin-39J Fab bound to cells expressing various I-A<sup>k</sup> molecules (M12.C3.F6, M12.C3.5D1 and M12.C3.411) are shown in Fig. 2. The traces indicate the substantial differences in class II expression that exist between clones expressing the various phenotypes. For example, M12.C3.411 cells expressing α-12/β-18 molecules completely lacking the cytoplasmic domain are expressed at a level only about one-fifth that of the αwt/βwt I-A<sup>k</sup> molecules on M12.C3.F6 cells and this is reflected in the phosphorescence intensities from these cells. M12.C3.F6 cells have one of the highest levels of class II expression observed, estimated at 2×10<sup>5</sup> copies/cell (26); however, this level is still appreciably lower than that of many other comparably studied membrane proteins, such as the type I Fce receptor on 2H3 cells. Thus time-resolved phosphorescence anisotropy measurements of class II rotation are always challenging—particularly so with poorly expressed phenotypes such as α-12/β-18. Phosphorescence decays for ErITC-39J anti-I-A<sup>k</sup> bound to the full range of cells studied are presented in Table 1. Time-resolved phosphorescence intensities were fitted to a decay model incorporating two exponentials plus a constant baseline, effectively a three-exponential model where the time constant of the third decay is arbitrarily long. As is typically observed for phosphorescence emission from
phosphorescence decay is kinetically similar for various and fully-truncated I-A k molecules in M12.C3 and K46J cells. A rapid component of ~20 µs with a fractional amplitude of ~0.2, a middle component of 100–150 µs with a fractional amplitude of ~0.6, and a constant component of amplitude ~0.2 were observed respectively. The fractional amplitude of ~0.2, a middle component of K46J. The phosphorescence half-times for the M12.C3 and K46J model was both necessary and sufficient differences.

**Table 1.** Phosphorescence lifetimes for ErITC–39J Fab bound to M12.C3 and K46J I-A k MHC class II truncates

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phenotype</th>
<th>Temperature (°C)</th>
<th>τ1 (µs)a</th>
<th>a1</th>
<th>τ2 (µs)b</th>
<th>a2</th>
<th>Constant</th>
<th>t1/2 (µs)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12.C3.F6</td>
<td>αwt/βwt</td>
<td>4</td>
<td>25.9 ± 0.1</td>
<td>0.19 ± 0.01</td>
<td>181.6 ± 0.4</td>
<td>0.72 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>104</td>
</tr>
<tr>
<td>4°C</td>
<td>αwt/βwt</td>
<td>4</td>
<td>18.3 ± 1.2</td>
<td>0.29 ± 0.01</td>
<td>133.6 ± 5.6</td>
<td>0.63 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>58</td>
</tr>
<tr>
<td>15°C</td>
<td>αwt/βwt</td>
<td>15</td>
<td>12.2 ± 0.2</td>
<td>0.22 ± 0.01</td>
<td>92.1 ± 1.3</td>
<td>0.61 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>57</td>
</tr>
<tr>
<td>25°C</td>
<td>αwt/βwt</td>
<td>25</td>
<td>12.0 ± 0.2</td>
<td>0.29 ± 0.01</td>
<td>80.7 ± 0.9</td>
<td>0.58 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>40</td>
</tr>
<tr>
<td>37°C</td>
<td>αwt/βwt</td>
<td>37</td>
<td>4.9</td>
<td>0.14</td>
<td>30.3</td>
<td>0.59</td>
<td>0.37</td>
<td>29</td>
</tr>
<tr>
<td>K46J.17.4</td>
<td>αwt/βwt</td>
<td>4</td>
<td>19.3 ± 0.4</td>
<td>0.29 ± 0.10</td>
<td>131.9 ± 3.3</td>
<td>0.62 ± 0.06</td>
<td>0.09 ± 0.04</td>
<td>59</td>
</tr>
<tr>
<td>M12.C3.5D1</td>
<td>α-6/βwt</td>
<td>4</td>
<td>22.3 ± 0.5</td>
<td>0.21 ± 0.07</td>
<td>166.1 ± 6.0</td>
<td>0.66 ± 0.08</td>
<td>0.13 ± 0.07</td>
<td>97</td>
</tr>
<tr>
<td>M12.C3.10B3</td>
<td>α-12/βwt</td>
<td>4</td>
<td>18.5 ± 2.4</td>
<td>0.17 ± 0.04</td>
<td>147.3 ± 21.7</td>
<td>0.66 ± 0.03</td>
<td>0.17 ± 0.03</td>
<td>100</td>
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<tr>
<td>M12.C3.5A2</td>
<td>αwt/β-12</td>
<td>4</td>
<td>25.9 ± 0.7</td>
<td>0.19 ± 0.01</td>
<td>178.0 ± 6.4</td>
<td>0.70 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>104</td>
</tr>
<tr>
<td>M12.C3.4B5.3</td>
<td>αwt/β-18</td>
<td>4</td>
<td>18.3 ± 0.7</td>
<td>0.24 ± 0.01</td>
<td>166.1 ± 1.6</td>
<td>0.65 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>85</td>
</tr>
<tr>
<td>M12.C3.5B2</td>
<td>α-12/β-12</td>
<td>4</td>
<td>18.6 ± 1.1</td>
<td>0.17 ± 0.03</td>
<td>147.9 ± 0.7</td>
<td>0.69 ± 0.09</td>
<td>0.14 ± 0.13</td>
<td>97</td>
</tr>
<tr>
<td>M12.C3.411</td>
<td>α-12/β-18</td>
<td>4</td>
<td>12.6 ± 0.0</td>
<td>0.24 ± 0.05</td>
<td>122.2 ± 0.9</td>
<td>0.60 ± 0.04</td>
<td>0.16 ± 0.01</td>
<td>72</td>
</tr>
<tr>
<td>K46J.7.1</td>
<td>α-12/β-18</td>
<td>4</td>
<td>17.3 ± 1.0</td>
<td>0.18 ± 0.14</td>
<td>144.0 ± 3.3</td>
<td>0.67 ± 0.15</td>
<td>0.15 ± 0.13</td>
<td>94</td>
</tr>
</tbody>
</table>

a Time constant followed by the fractional amplitude.
b Overall phosphorescence half-time t1/2 calculated from indicated mean decay times and amplitudes.

The phosphorescence lifetime with increasing temperature, this quantity decreasing from 58 µs for wild-type I-A k in M12.C3 cells at 4°C to 30 µs at 37°C.

**Wild-type I-A k rotational and lateral diffusion resembles that of other MHC class II molecules and is similar in M12.C3 and K46J cells**

The upper panel of Fig. 3 shows a representative phosphorescence anisotropy decay curve for a M12.C3.F6 cell expressing αwt/βwt class II. Results of time-resolved phosphorescence anisotropy measurements at 4°C for M12.C3 and K46J αwt/βwt molecules are presented in Table 2. Rotational correlation times were 11.9 and 10.9 µs respectively, with initial and limiting anisotropy values for each cell type of ~0.044 and 0.040 respectively. The rotational correlation times are not statistically different between these two cell types, suggesting that the rotating unit containing class II molecules is approximately the same in both cell types. The phosphorescence t1/2 values calculated from mean decay parameters (Table 1), however, do appear to be different for the wild-type M12.C3 and K46J cell types, where t1/2 values are 104 and 59 µs respectively. This would suggest that erythrosin labels have greater accessibility to oxygen when 39J Fab is bound to K46J cell I-A k.

For the other class II phenotype examined on both M12.C3 and K46J cell types, i.e. α-12β-18, rotational parameters in both cell types were also similar, though rotational correlation times were substantially faster than those observed for wild-type I-A k. Cell lines expressing α-12β-18 molecules exhibit rotational correlation times of 7.2 µs in M12.C3 and 6.8 µs in K46J. The phosphorescence half-times for the M12.C3 and K46J fully truncated cell types were similar at 72 and 94 µs respectively.

The average lateral diffusion coefficients for the wild-type and fully-truncated I-A k molecules in M12.C3 and K46J cells are presented in Table 4, whose other contents will be presented later. Each cell type exhibited diffusion coefficients of ~2×10⁻⁹ cm²/s with fractional mobilities about 60% at 23°C. There do not appear to be significant differences.
in lateral diffusion coefficients or percent mobile fractions between the M12.C3 and K46J cell types, as was also indicated by the rotational diffusion results. The rotational and lateral diffusion values presented here are typical of other wild-type MHC class II molecules as measured under similar conditions (9,28).

**Rotational and lateral motions of truncated I-A^K**

**Table 2.** Rotational diffusion parameters for ErITC-39J Fab bound to M12.C3 and K46J I-A^K MHC class II truncates

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phenotype</th>
<th>Temperature (°C)</th>
<th>$\langle \mu s \rangle^a$</th>
<th>$t_0^a$</th>
<th>$r_\alpha^a$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12.C3.F6</td>
<td>αwt/βwt</td>
<td>4</td>
<td>11.9 ± 2.6</td>
<td>0.044 ± 0.006</td>
<td>0.041 ± 0.006</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>10.4 ± 1.2</td>
<td>0.037 ± 0.001</td>
<td>0.032 ± 0.001</td>
<td>1</td>
</tr>
<tr>
<td>K46J.17.4</td>
<td>αwt/βwt</td>
<td>4</td>
<td>10.9 ± 4.2</td>
<td>0.044 ± 0.010</td>
<td>0.043 ± 0.009</td>
<td>11</td>
</tr>
<tr>
<td>M12.C3.5D1</td>
<td>α-6/βwt</td>
<td>4</td>
<td>4.8 ± 1.1</td>
<td>0.044 ± 0.005</td>
<td>0.041 ± 0.003</td>
<td>13</td>
</tr>
<tr>
<td>M12.C3.10B3</td>
<td>α-12/βwt</td>
<td>4</td>
<td>15.5 ± 8.0</td>
<td>0.043 ± 0.003</td>
<td>0.041 ± 0.003</td>
<td>25</td>
</tr>
<tr>
<td>M12.C3.5A2</td>
<td>α-6/β-12</td>
<td>4</td>
<td>9.2 ± 2.3</td>
<td>0.040 ± 0.003</td>
<td>0.035 ± 0.003</td>
<td>24</td>
</tr>
<tr>
<td>M12.C3.4B5.3</td>
<td>α-6/β-18</td>
<td>4</td>
<td>10.4 ± 1.0</td>
<td>0.047 ± 0.012</td>
<td>0.046 ± 0.013</td>
<td>15</td>
</tr>
<tr>
<td>M12.C3.5B2</td>
<td>α-12/β-12</td>
<td>4</td>
<td>10.1 ± 1.9</td>
<td>0.045 ± 0.003</td>
<td>0.041 ± 0.003</td>
<td>25</td>
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<tr>
<td>M12.C3.411</td>
<td>α-12/β-18</td>
<td>4</td>
<td>7.2 ± 3.7</td>
<td>0.031 ± 0.004</td>
<td>0.029 ± 0.002</td>
<td>6</td>
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<tr>
<td>K46J.7.1</td>
<td>α-12/β-18</td>
<td>4</td>
<td>6.8 ± 2.3</td>
<td>0.046 ± 0.005</td>
<td>0.043 ± 0.004</td>
<td>15</td>
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</tbody>
</table>

$^a$Mean ± SD for $n$ measurements of rotational correlation time, initial anisotropy $t_0$, and limiting anisotropy $r_\alpha$.

$^b$Cells labeled with 20 μM ErITC-intact 39J mAb for 30 min on ice.

The rotational diffusion parameters for 39J Fab-labeled cells expressing I-A^K molecules with various cytoplasmic truncations are presented in Table 2. After labeling with aggregate-free probes, most I-A^K molecules, wild-type and structurally modified, appear to be substantially mobile rotationally with rotational correlation times of ~10 μs, and initial and limiting anisotropies of ~0.043 and 0.040 respectively. Cells expressing the α-12/β-18 phenotype exhibited the fastest rotational correlation times relative to wild-type I-A^K, i.e. 7.2 ± 3.7 and 6.8 ± 2.3 μs for M12.C3 and K46J α-12/β-18 transfectants respectively. One discordant rotational correlation time is exhibited by the α-6/βwt phenotype with a rotational correlation time of 4.8 ± 1.1 μs. This 2-fold increase in rotational diffusion over wild-type molecules is not apparent, however, in other cells that also express I-A^K with truncations of the first six amino acid residues from the α chain, such as α-12/βwt and α-12/β-12.

The initial anisotropy and limiting anisotropy values measured for each of the nine truncates are quite similar as shown in Table 2. These results are averaged initial and limiting phosphorescence anisotropy values obtained over 3–5 different days. The anisotropy decay traces for M12.C3 cells expressing αwt/βwt, α-6/βwt and α-12/β-18 molecules are presented in Fig. 3. The phosphorescence decay half-times also do not differ significantly among the M12.C3 truncated I-A^K molecules. The half-times ranged from 60 to 100 μs. There was no trend or significant changes observed between the various I-A^K truncates.

**FBR measurements of MHC class II translational diffusion**

are consistent from day to day and among the methods and probes

Previous measurements of lateral diffusion for truncated I-A^K molecules demonstrated substantial variation from day to day (10). Thus it was somewhat surprising to find that, when we repeated these measurements using both spot and fringe FPR, we found excellent consistency from day to day as well as between methods and labels. These results are presented in Table 3. The rate of lateral diffusion $D$ and the percent mobility %M measurements for 39J intact mAb and Fab labeled transfected M12.C3 and K46J cells yields results typically consistent from day to day. For example, the standard deviations of the daily average diffusion coefficient and percent mobility for Fab-labeled M12.C3.F6 (αwt/βwt) cells are ±0.4 x 10^{-10} cm^2/s in $D$ and ±5% in %M for the fringe FPR experiments. This standard deviation is obtained from three different days of experiments with 15 individual cells analyzed per day. Thus, the day-to-day variation is <10% for both $D$ and %M with a few of the poorly expressing cell lines exhibiting somewhat larger variation. The higher day-to-day variation in the diffusion parameters of the poorly expressed I-A^K phenotypes, α-12/β-18, α-12/βwt and αwt/β-18, is apparently due to the decreased signal-to-noise ratio obtained in FPR measurements on these cells.

Both spot and fringe FPR methods yield statistically comparable results for I-A^K lateral diffusion coefficient on cells labeled with either intact 39J mAb or Fab as shown in Table 3. Table 4 shows the average diffusion coefficient and fractional mobility as measured in four independent experimental situations for each truncate listed in Table 3 since there was no statistically significant variation among the methods or labels. Table 3 shows the diffusion coefficient and the fractional mobility for each of the nine I-A^K truncated cell lines arranged by phenotype. Each column presents the diffusion coefficient and the percent mobility for I-A^K on cells labeled with intact 39J mAb or Fab measured with either the fringe or spot photobleaching method. While no variations in diffusion coefficient were observed for the fringe and spot methods, some slight differences in mobile fraction were noted, however. For example, there is a slight, but not statistically significant, increase in the average fractional mobility measured with the fringe method for I-A^K molecules labeled with 39J Fab of 76 ± 15%, when compared to I-A^K molecules measured with the spot method for cells labeled with 39J mAb (49 ± 11%) and Fab (49 ± 13%).
Deletions in the cytoplasmic domain of I-A<sup>k</sup> have limited effects on measured I-A<sup>k</sup> lateral diffusion coefficients

As amino acid residues were progressively deleted from the α and β chain cytoplasmic tails of I-A<sup>k</sup>, the rate of lateral diffusion did not change significantly. The diffusion coefficients of all truncates lie within 1–2 SD of one another from αwt/βwt down to α-12β-18. Representative fringe fluorescence photobleaching recovery traces for Fab-labeled M12.C3.F6 (αwt/βwt), M12.C3.5D1 (α-6β/β) and M12.C3.411 (α-12β-18) cells are presented in Fig. 4. All cell lines exhibit approximately the same diffusion parameters, i.e. a diffusion coefficient of ~2 × 10<sup>–10</sup> cm<sup>2</sup>/s and a fractional mobility of 60–70%. The 2-fold increased rotational rate of α-6β/βwt molecules was thus not apparent in the lateral diffusion measurements.

**Discussion**

Comparison of the rotational and translational diffusion data from this study for the I-A<sup>k</sup> truncates suggests that these...
molecules exhibit motional freedom typical of many membrane proteins. Previous studies of the molecular motions of wild-type I-A^k molecules (9), and other similar transmembrane proteins such as H-2K^k antigen (30), Fc receptor type I (27) and luteinizing hormone receptor (31) show rotational correlation times of each protein of 10-20 ms and lateral diffusion coefficients in the range of 2-5×10^{-10} cm^2/s with fractional recoveries of ~50%. The rotational correlation times and diffusion parameters reported in this study are therefore typical of rotationally and translationally mobile cellular proteins.

Previous experiments have suggested that α-12/β-18 molecules exhibit rates of lateral diffusion perhaps 10-fold faster than the wild-type (10). Thus, substantially more rapid rotational diffusion might be expected for the α-12/β-18 protein. The M12.C3 and K46J α-12/β-18 phenotypes did in fact exhibit a 2-fold smaller average rotational correlation time compared to the wild-type molecules and this may indicate a decrease in I-A^k associations with other molecular species after complete deletion of the cytoplasmic domains. Enhanced lateral diffusion previously reported for the α-6/β wt and α-12/β-18 molecules suggested that these species might also exhibit rotational diffusion coefficients substantially higher than the wild-type. In fact, the α-6/β wt molecules did show a rotational correlation time ~2-fold faster than the wild-type molecules. This might be taken as reflecting loss of interactions involved in antigen presentation (6) since the α chain cytoplasmic domain has been implicated in both these functions. However, this faster rotational correlation time is probably peculiar to this particular cell line since the α-12/β wt and the α-12/β-18 molecules did not show the same increase in rotational correlation time. Partial deletions of cytoplasmic amino acids from the α and β chains of the I-A^k molecules thus only slightly altered the rotational diffusion of the class II molecule within the plasma membrane.

Phosphorescence lifetime values observed for the truncates also did not differ significantly among the truncates except for the K46J wild-type cell line. The almost 2-fold shorter t_{1/2} measured for the wild-type K46J cell line over the wild-type M12.C3 cell line may indicate that the M12.C3 class II complex does not allow as much oxygen, known to quench the phosphorescence triplet state, into the class II complex. This suggests that the I-A^k complex on unperturbed M12.C3 cells might be larger or more tightly packed than on K46J cells. This would agree with known functional behavior of these cell types since PKC and cAMP signaling by M12.C3 cells requires only dimerization of class II while Ca^{2+} signaling by K46J cells requires extensive cross-linking of class II. In K46J transfecteds that express either wild-type or α-12/β-18 class II molecules, the molecular motions of the wild-type compared to the fully truncated class II are not different. This is not unexpected as the ability to activate tyrosine kinases that mediate subsequent Ca^{2+} flux does not require the cytoplasmic domains (12) or the transmembrane region of class II (W. F. Wade and J. Cambier, pers. commun.).

The lateral diffusion coefficients and fractional mobilities observed in this study for I-A^k are typical of the values of 2×10^{-10} cm^2/s that have been reported previously for I-A^d (9) and for wild-type I-A^k (10,32). When MHC class II molecules are labeled with 39J Fab fragments, a slightly higher percent-

![Fig. 4](image-url) Representative fringe FPR traces for individual TAMRA-39J anti-α^k Fab-labeled M12.C3.F6 (αwt/βwt), M12.C3.5D1 (α-6/β wt) and M12.C3.411 (α-12/β-18) cells at 23°C. All three transfectants exhibit an I-A^k lateral diffusion coefficient of 1.7×10^{-10} cm^2/s. Percent mobilities are as follows: αwt/βwt, 73%; α-6/βwt, 59%; and α-12/β-18, 64%.

![Fig. 5](image-url) Increasing the laser power during spot FPR measurements results in an apparent increase in the rate of lateral mobility of fully truncated MHC class II molecules at 23°C. The upper plot in the graph (●) shows that the diffusion coefficient of α-12/β-18 I-A^k on M12.C3.411 cells increases markedly as probe power is increased. The lower plot in the graph (▲) shows wt/wt I-A^k lateral diffusion on M12.C3.F6 cells to be quite insensitive to increasing probe beam power. Error bars indicate the SEM of the lateral diffusion coefficient measured at each power.
age of the molecules appear mobile (e.g. 76 ± 15% in the fringe FPR measurements of F6 cells) than when intact mAb is used (57 ± 5%). This could be explained if intact 39J mAb cross-links I-A^k molecules into antibody-linked pairs of heterodimers. Apart from these slightly higher percent mobilities consistently measured for Fab-labeled cells, there remain no significant differences or notable trends of changing translational or rotational molecular dynamics among the nine MHC class II truncates.

Despite the known involvement of cytoplasmic residues in various aspects of class II signaling, truncating I-A^k cytoplasmic domains seems to have limited effect on class II lateral diffusion coefficients. The most unexpected result of this study was our failure to find significant correlation between class II molecules with cytoplasmic truncations and enhanced lateral mobility. Only one experimental factor differs between earlier and current measurements, and so might be suspected of occasioning this discrepancy. All FPR experiments presented here were performed at low and consistent laser power densities of 1.4 μW/μm^2. Under these conditions, irreversible photobleaching of labeled molecules in the probe beam is <10% during a 30 s experiment. Previous experiments on this system apparently involved higher probe beam powers with necessarily higher rates of label photobleaching. Higher probe beam power has differential effects according to the expression level of the protein examined. In the laser power experiments presented in Fig. 5, as the laser powers were increased to enhance fluorescence signals, the apparent rate of α-12β-18 lateral diffusion increased by 8-fold over the wild-type I-A^k molecules and lateral diffusion of partially truncated class II molecules fell roughly on the same regression line according to their surface expression. The question of how FPR measurements using high power levels on cells expressing low densities of labeled receptors might yield rapid diffusion parameters is puzzling. One possible explanation could be cellular autofluorescence which is more resistant to photobleaching than are chromophores like rhodamine and fluorescein. If a weakly labeled cell is examined microscopically under a strong probe beam for a few seconds before a bleach is performed, the fluorescent label is substantially depleted while the contribution from the autofluorescence is less affected. Increasing powers used in a consistent measurement protocol thus increasingly reflect the diffusion of autofluorescence cytoplasmic species but the relative magnitude of the effect depends upon the initial concentration of chromophores on the cell surface. Since M12.C3.411 cells of the α-12β-18 phenotype have only 20% of the I-A^k expression of M12.C3.F6 (αwt/βwt) cells, one might expect that high-power observation of both cell types could lead to very fast apparent diffusion for M12.C3.411 cells only.

The data we present have important implications for the structure of functional class II complexes on cell surfaces. The molecular dynamics of the truncated I-A^k molecules are only modestly affected by the cytoplasmic domains of the α and β chains. Yet these domains are known to participate in various aspects of class II function. Several possibilities could explain the relatively small effects of truncating the cytoplasmic tails of MHC class II molecules on the apparent lateral and rotational dynamics of these molecules. First, accessory molecules might always be bound to MHC class II with actual signaling arising from a conformational change of the complex dependent upon the cytoplasmic tail. In this case the overall physical changes associated with actual class II signaling might be quite subtle biophysically but profound in terms of biological interactions and so only be apparent by loss of signaling function. A signaling complex involving class II seems to be at least partially preformed in M12.C3 cells since class II ligation with only a single mAb is adequate to cause PKC translocation (33). Second, the functional class II complex might be sufficiently large that association or dissociation of some individual signaling molecules make only small unmeasurable changes in the complex’s overall dynamics. MHC class II molecules within the membrane of B lymphocytes have been shown through various means to be in close proximity to the IL-2 receptor α subunit (34), ICAM-1 proteins (34), MHC class I molecules (34,35) and TAP-1 (36), and possibly other MHC class II molecules (35,37). Constant class II interaction with other membrane proteins involved in B cell activation, such as those listed above, might mask whatever changes in the molecular dynamics I-A^k molecules truncation of cytoplasmic domains might cause. Third, interactions of accessory molecules with MHC class II and subsequent signaling may be sufficiently dependent upon TCR or antibody engagement or class II clustering so as not to be apparent in studies of unperturbed cells using Fab labels. This situation could arise in K46J cells where extensive class II cross-linking is required for signaling and treatment with even intact mAb is insufficient, a cross-linking reagent being necessary (12). Such a requirement for extensive cross-linking could explain our observation that photobleaching recovery and time-resolved phosphorescence anisotropy measurements using 39J intact mAb probes show no larger effects of truncation than do studies using Fab labels since intact 39J may produce insufficient cross-linking to recruit accessory molecules not already in the complex. A final possibility is the interactions involve only a subpopulation of class II molecules. If only 10% of class II molecules interact through cytoplasmic domains with signaling proteins, it is doubtful that the presence or absence of such interactions could be assessed by the dynamic measurements employed. For example, this subpopulation might be I-A^k dimer of dimers species which, if they exist on unperturbed B cells, apparently represent only a fraction of class II molecules. One might suppose that these possibilities apply variously to different cell types as, for example, to the M12.C3 and K46J cells cited above.

Acknowledgments

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Abbreviations

ErITC erythrosin isothiocyanate
FPR fluorescence photobleaching recovery
PKC protein kinase C
TAMRA [6-[tetrakis(methylrhodamine-5(6))-carboxamido]hexanoic acid succinimidyl ester

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### References