Modulation of CD4 lateral mobility in intact cells by an intracellularly applied antibody

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This study shows that the lateral mobility of CD4, an important plasma-membrane immune receptor, can be modulated by intracellular application of an anti-CD4 antibody. For this purpose, (i) full-length CD4 and a truncated CD4 mutant, lacking a 32-residue-long C-terminal intracellularly exposed domain, were expressed in *Spodoptera frugiperda* (Sf9) insect cells, (ii) a monoclonal antibody, C6, with specificity for the C-terminal domain was generated, and (iii) a versatile apparatus for fluorescence microphotolysis (FM) studies was constructed. By these means it was found that the commercial anti-CD4 antibody Leu3a-PE, in contrast with several other anti-CD4 antibodies, could be used as a fluorescent label of CD4 without interfering

greatly with CD4 mobility. Labelled by Leu3a-PE, full-length CD4 had a lateral diffusion coefficient of $D = (4.7 \pm 1.9) \times 10^{-10}$ cm²/s and a mobile fraction of $f_m = 80 \pm 16\%$ (room temperature). Within experimental accuracy the truncated CD4 had the same mobility as full-length CD4. Introduction of the C6 antibody into Sf9 cells by microinjection or by fusion with C6-loaded liposomes decreased the mobility of full-length CD4 ($f_m = 40\%$) but not of truncated CD4 ($f_m = 80\%$). Treatment of Sf9 cells with phorbol ester also reduced the mobility of full-length CD4 ($f_m = 50\%$) but not truncated CD4 ($f_m = 90\%$). A calmodulin inhibitor but not a protein kinase C (PKC) inhibitor abolished the phorbol ester effect.

INTRODUCTION

CD4, a 55 kDa transmembrane glycoprotein, is an important immune receptor cooperating with the T-cell receptor in binding the major histocompatibility complex class II of antigen-presenting cells [1–3]. The cytoplasmic domain of CD4 interacts with p56^{lck}, a tyrosine protein kinase [4], indicating that CD4 is also involved in the transmission of differentiation signals. In addition, CD4 is a receptor for gp120 [5,6], the glycoprotein of the human immunodeficiency virus (HIV) [7–10]. The interaction between CD4 and gp120 initiates the infection process and induces the fusion of the HIV envelope with the T-cell host [11,12].

The dynamics of CD4 has important physiological implications. Thus the clustering of CD4, which can be induced by sequential reaction with a monoclonal anti-CD4 antibody and a polyclonal anti-immunoglobulin antibody, causes CD4 to interact with and thus to activate p56^{lck} [13] and can also induce the endocytosis of CD4 [13,14]. In non-lymphoid cells the endocytosis of CD4 proceeds via coated pits [15,16] and is followed by digestion in lysosomes.

Dynamic processes of membrane receptors, such as lateral mobility, aggregation, clustering and endocytosis, have frequently been studied by mobility measurements involving the FM method [17–22]. However, in previous studies the modulation of receptor dynamics was most frequently induced from the external cell surface, i.e. by adding ligands or antibodies to the medium. In this study we explored whether the mobility of CD4

can be specifically modulated from the cell interior, namely by reaction with an antibody specific for a cytoplasmic receptor domain. Therefore we generated an antibody specific for an intracellularly exposed CD4 domain of full-length CD4 and set up a versatile FM apparatus. Moreover, Sf9 insect cells were used as a cellular model system because this permitted us to express not only full-length CD4 but also a CD4 mutant lacking the epitope recognized by the intracellularly applied antibody. The validity of Sf9 cells as a model system was strengthened by the observation that phorbol 12-myristate 13-acetate (PMA) apparently induced the endocytosis of wild-type CD4, similarly to reports [23,24] for mammalian lymphoid and non-lymphoid cells.

By these means we could show for the first time that the lateral mobility of a membrane-localized receptor can be modulated in the living cell by intracellular application of a receptor-specific antibody.

MATERIALS AND METHODS

Construction of baculovirus expression vectors for full-length CD4 and a C-terminally truncated CD4 mutant

DNA manipulation and construction of baculovirus expression vectors were carried out according to standard procedures [25,26]. R. Axel (Howard Hughes Medical Institute, Columbia University, New York, NY) kindly provided the human CD4 sequence in the form of plasmid pT4B [27,28]. The baculovirus

Abbreviations used: D, lateral diffusion coefficient; FITC, fluorescein isothiocyanate; f_m , mobile fraction; FM, fluorescence microphotolysis; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; PMA, phorbol 12-myristate 13-acetate; PE, phycoerythrin; PKC, protein kinase C; Sf9, *Spodoptera frugiperda*; W7, N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide.

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transfer vector pv941 [26] was used in the construction of both the full-length CD4 and the C-terminally truncated mutant expression vectors. The construction of the baculovirus expression vectors for full-length CD4 (pAc-CD4) has been described previously [29]. This vector was modified as follows to produce the C-terminally truncated mutant: pAc-CD4 was digested with the restriction enzymes *NarI* and *EcoRV* to release a CD4 coding sequence lacking nucleotides from the C-terminal (cytoplasmic) portion of the gene. This fragment was inserted into the plasmid pBAC-1511 (a gift from Y. Gluzman, Cold Spring Harbor Laboratory), using an *NarI/EcoRV*-compatible synthetic linker containing a stop codon and used as an adapter. Thus the full-length CD4 cDNA contained coding information for 433 amino acids, with amino acids 373–393 corresponding to the transmembrane region and amino acids 394–433 corresponding to the cytoplasmic tail. The C-terminal modification resulted in the deletion of the last 31 amino acids from the cytoplasmic tail. Isolated full-length and truncated CD4 were identified in 10% SDS/PAGE (Bio-Rad, Hercules, CA, U.S.A.) and stained with Coomassie Blue.

Culturing of insect Sf9 cells and their infection with baculovirus

Insect Sf9 cells were obtained from Texas A&M University, Department of Entomology (College Station, TX 77845, U.S.A.) and cultured as previously described [29,30]. Briefly, cells were cultured in Ex-cell 400 medium (JRH Biosciences, Lenexa, KS, U.S.A.) at 27 °C in suspension culture to a density of $(2-3) \times 10^6$ cells/ml. For infection with the baculovirus, 40 ml of Sf9 insect cells at 2×10^6 cells/ml, 10 ml of Ex-cell 400 medium and 10 ml of baculovirus with a titre of about 2.0×10^9 plaque-forming units per ml [30] were mixed in a spinner flask and cultured for 1 day at 27 °C.

Flow cytometric determination of CD4 in the plasma membrane of intact Sf9 cells

The relative amount of CD4 in the plasma membrane of Sf9 cells was measured 24 h after infection by using a flow cytometer (Coulter Epics, Miami, FL, U.S.A.). Sf9 cells were labelled with a saturating concentration of anti-Leu3a-phycoerythrin (PE) (Becton Dickinson, San Jose, CA, U.S.A.), as previously described [30]. In general, 300 000 cells were pelleted in an Eppendorf microcentrifuge (15 s, 14 000 rev./min). The pellet was resuspended in 50 μ l fresh Ex-cell 400 medium containing 0.6 μ g anti-Leu3a-PE. After 20 min of incubation at 4 °C, 250 μ l of fresh Ex-cell 400 medium was added and the fluorescence measured in a flow cytometer. For all photobleaching experiments reported here, Sf9 cells in the early logarithmic phase of infection were used.

Generation and characterization of the monoclonal C6 antibody

The C6 antibody was produced by Organon Teknika Biotechnology Research Institute (Rockville, MD, USA) as an IgG_{2b} mouse isotype and stored in a stock solution of 2.7 mg/ml. Using a modification of the method of Butman et al. [31], the antibody was raised against an oligopeptide antigen with the following sequence: Arg¹-Arg-Gln-Ala-Glu-Arg-Arg-Nle-Ser-Gln¹⁰-Ile-Lys-Arg-Arg-Gly-Gly¹⁸. This is a modified sequence of human CD4 [27,28], residues 400–412. Met⁴⁰⁷ of the human CD4 sequence is replaced with Nle⁸ to prevent interference in the coupling reaction with BSA and the last three amino acids (-Arg-Ala-Gly-) are added to aid BSA coupling. This oligopeptide was used for immunization, and hybridomas were produced.

Hybridomas were screened by indirect ELISA for reactivity against CD4. Specificity of hybridomas for the unconjugated CD4 peptide was confirmed by indirect dot-blot assay. Selected hybridomas were chosen by two further rounds of limiting dilution screening. Monoclonal antibody isotypes were determined by double immunodiffusion. Subclones were propagated as ascitic tumours and the monoclonal antibodies were purified by affinity chromatography over Protein A-Sepharose. The purified monoclonal antibodies were dialysed against PBS, filter-sterilized, frozen and stored at -70 °C. Non-specific IgG_{2b} was obtained from Sigma (St. Louis, MO, U.S.A.).

Infected Sf9 cells were used for CD4 isolation as previously described [29]. Isolated full-length and truncated CD4 was blotted on nitrocellulose membrane (BioRad, Bio-Dot, Hercules, CA, U.S.A.). The membrane was blocked with 3% (w/v) gelatin for 30 min at 25 °C. Leu3a antibodies at a concentration of 300 ng/ml or 8 nmol of lipid per ml, as C6-antibody encapsulated in vesicles, were incubated for 2 h (25 °C) in a 0.05% Tween-20-containing PBS buffer. Mouse alkaline phosphatase conjugate was used from a BioRad detection kit as secondary antibody for 2 h at 25 °C and the blot was developed as described by the manufacturer.

The specificity of C6 was also tested with permeabilized Sf9 cells. For this purpose baculovirus-infected Sf9 cells were permeabilized with 70% (v/v) ethanol for 8 min at -20 °C. Cells were washed twice with PBS and then incubated with 50 μ l C6 antibody (2 mg/ml) for 20 min on ice. After twice being washed with PBS, the cells were incubated with rabbit anti-mouse IgG-FITC antibodies (Jackson Immuno Research, West Grove, PA, U.S.A.) at 10 μ g/ml for 20 min on ice. Cells were washed twice, resuspended in 300 μ l, and the fluorescence was detected by flow cytometry. Control cells were labelled after permeabilization with non-specific IgG_{2b} and with the secondary antibody.

Preparation of specimens for microinjection and photobleaching measurements

Special precautions were taken to minimize mechanical stress of infected cells during the labelling and washing procedures. Infected Sf9 cells were plated in growth medium on 18 mm $\times$ 12 mm coverslips and allowed to attach firmly to the glass surface for at least 1 h at 27 °C. A cover slip with adhering cells was then taken, surplus medium removed and incubated with 5 μ l of undiluted Leu3a-PE solution (Becton Dickinson, San Jose, CA, U.S.A.). In some experiments, instead of Leu3a-PE the antibodies Leu3a-FITC, OKT4-FITC or OKT4A-FITC were used. After 5 min at room temperature, the cover slip was placed into a Petri dish suitable for microscopy (Petritherm; Heraeus, Hanau, Germany), containing about 8 ml of growth medium. Labelling with goat anti-mouse antibodies (Pierce, Rockford, IL, U.S.A.; 1.5 mg/ml) against Leu3a was done under the same conditions.

Construction of a photobleaching apparatus combining mobility measurements with microinjection

The photobleaching apparatus was constructed with an inverted microscope, following established principles [32]. However, to improve the stability, sensitivity and accuracy of photobleaching measurements, and also to meet the particular requirements of the present study, a number of modifications were introduced. Fibre optics were employed to guide the laser beam to the microscope. A comprehensive computer program (SuperScope II; GW Instruments, Somerville, MA, U.S.A.) was used to

control the measuring and data accumulation process. The microscope was equipped with a microinjection set-up and with optics (40-fold, N.A. 0.60, long-distance objective lens) permitting both photobleaching measurements and microinjection. Thus lateral mobility could be measured on the same cells before and after microinjection.

Mobility measurements and data evaluation

For mobility measurements a Petri dish containing a cover slip with adherent labelled cells (see above) was placed on the stage of the microscope. The surface of a cell was brought into focus and the plasma membrane homogeneously illuminated within a circular area of 1.8 μm diameter. Fluorescence was recorded for 20 s, employing a laser beam power (~ 100 nW in the sample) that did not induce a significant degree of photobleaching during the total measuring time of 170 s. After 20 s the beam power was increased to approximately 1 mW for about 50 ms, which decreased the fluorescence of the illuminated area by 60–80 %. Fluorescence was then continuously recorded for another 150 s at the initial small beam power. The experimental data were evaluated according to the concepts of Axelrod et al. [20] and Soumpasis [33], which hold for two-dimensional isotropic lateral diffusion of a mobile and an immobile species (in this context 'immobile' means that the diffusion coefficient is equal to or less than 2×10^{-12} cm^2/s). The experimental data were analysed for the lateral diffusion coefficient D and the mobile fraction f_m by fitting them to the appropriate theoretical expression by using a non-linear least-squares procedure based on the Levenberg–Marquart method.

Microinjection of Sf9 cells

Microinjections were performed with a model NO-203 micro-manipulator and model TM-200 microinjector, both from Narashige (Greenvale, NY, U.S.A.). Micropipettes (Femtotips) were obtained from Eppendorf (Hamburg, Germany). These were filled with a solution of either C6 or 13.B.8.2 (AMAC, Westbrook, ME, U.S.A.), both antibodies having a concentration of ~ 2.0 mg/ml in the injected solution. A small amount of FITC-labelled 20 kDa dextran (final concentration about 0.1 mg/ml) (Sigma, St. Louis, MO, U.S.A.) was added to the injection solutions. Cells were selected and the micropipette was adjusted using phase contrast. The actual injection was performed under fluorescence illumination, which permitted direct observation of the introduction of fluorescent material into the cell. The injected fluorescence did not influence the mobility measurements, and the fluorescence intensity of the cell surface was approximately the same before and after injection as measured by FM. The first mobility measurement was always made before injection. Only the cells that did not show any changes detectable by fluorescence and phase contrast observation were subjected to a second mobility measurement, about 10 min after injection.

Delivery of the C6 antibody and non-specific IgG2b antibody to Sf9 cells by liposomes

Liposomes were prepared from a mixture of 5 μmol 1,2-dioleoyl-3-trimethylammonium propane and 5 μmol of 1,2-dioleoyl-*sn*-glycerol 3-phosphoethanolamine. They were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and made by reverse-phase evaporation, as described by Szoka and Papahadjopoulos [34] and Düzgünes et al. [35], using multiple extrusions through two stacked 0.08 μm polycarbonate membranes (Poretics, Livermore, CA, U.S.A.) with a hand-held

extrusion device (Avestin, Ottawa, Canada). Liposomes were prepared in 150 mM NaCl, 10 mM Hepes, 0.1 mM EDTA, pH 7.4. Liposomes for antibody delivery were prepared with 2.0 mg/ml of antibodies, and were used without separation of unencapsulated material. Cells (2.0×10^5 per ml, in PBS) were labelled with 10 nM of lipid for 10 min at 4 °C. Washing and culturing of cells was as described before.

Incubation of Sf9 insect cells with PMA, W7 and H7

Leu3a-PE-labelled Sf9 insect cells were incubated with 25 μM W7-calmodulin inhibitor [*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide] or 25 μM H7-PKC inhibitor [1-(5-isoquinolinesulphonyl)-2-methylpiperazine] for 30 min at 27 °C (both from Calbiochem, San Diego, CA, U.S.A.). PMA was added to a final concentration of 100 nM and diffusion of CD4 in the plasma membrane of Sf9 insect cells was measured 30 min later.

RESULTS

Specificity of the newly generated monoclonal antibody, C6

The specificity of C6, a monoclonal antibody directed against the C-terminal, intracellularly exposed residues 400–412 of human CD4 (see Materials and Methods), was studied with isolated full-length or truncated CD4 (Figure 1). A comparison of C6 with Leu3a, a commercial monoclonal antibody specific for an extracellular epitope of CD4, in dot-blot experiments showed that full-length CD4 was recognized by both antibodies with similar affinity (Figure 2a). Truncated CD4, however, was only recognized by Leu3a but not C6 (Figure 2b). We also established that encapsulation of C6 by liposomes did not interfere with its binding capacity and its specificity (Figure 2b). Further, the binding of C6 to CD4 contained in the plasma membrane of Sf9 cells was tested. For this purpose infected Sf9 cells, expressing full-length or truncated CD4, were permeabilized with 70 % (v/v) ethanol and then labelled with C6 primary antibodies followed by a FITC-conjugated secondary antibody. It was found (Figure 3) that C6 bound only to Sf9 cells expressing full-length CD4 (Figure 3a), not to cells expressing truncated CD4 (Figure 3b).

Figure 1 Gel electrophoretogram of isolated full-length CD4 and the truncated CD4 mutant

SDS/PAGE (10%) of 10 μg of full-length CD4 (lane 1), 10 μg of truncated CD4 mutant (lane 2) and molecular mass marker (lane 3), stained with Coomassie Blue.

Figure 2 Specificity of the C6 antibody

Dot-blot are shown of (i) truncated CD4 and (ii) full-length CD4 employing 1000 ng (lane 1), 500 ng (lane 2), 100 ng (lane 3), 50 ng (lane 4) or 10 ng (lane 5) of CD4. Proteins were detected (a) with Leu3a (300 ng/ml in 0.05% Tween buffer), a commercial antibody specific for a cytoplasmic CD4 domain, and (b) with the C6 antibody, encapsulated in vesicles [8 nM of lipid (C6-antibody) per ml of 0.05% Tween buffer]. Blots were developed with a kit using alkaline phosphatase-conjugated antibodies (BioRad).

Figure 3 Binding of C6 antibody to CD4 exposed on the plasma membrane of Sf9 cells

Sf9 cells were infected with the baculovirus vector for full-length CD4 (a) or truncated CD4 (b). Infected cells were permeabilized and labelled with C6 (broken line) or a non-specific mouse IgG (solid line) and subsequently with rabbit anti-mouse-FITC antibodies. The flow cytometric curves show that substantial binding of C6 was observed only with permeabilized cells expressing full-length CD4.

Fluorescent labelling of CD4 in the plasma membrane of Sf9 cells

For fluorescence microscopy studies and most of the mobility measurements described below, infected Sf9 cells were labelled with Leu3a-PE. The specificity of Leu3a labelling was apparent from experiments with non-infected cells, which displayed

virtually no fluorescence after Leu3a-PE labelling (Figures 4e and 4f). In cells expressing either full-length or truncated CD4, labelling with Leu3a-PE resulted in a ring-like fluorescence which was largely homogeneous, although brighter punctate regions superimposed on a homogeneous background could be frequently recognized (Figures 4a–4d). This appearance did not change when the cells were kept at room temperature for several hours. Among different cells, the peripheral fluorescence varied within wide limits. In addition to Leu3a-PE, the fluorescent monoclonal antibodies Leu3a-FITC, OKT4-FITC and OKT4A-FITC were employed as markers of CD4. All of these antibodies are specific for extracellular epitopes of CD4. In contrast to Leu3a-PE, however, they were conjugated with FITC, a fluorophore that is very much smaller (389 Da) than PE (140 kDa). Upon labelling of CD4 expressing Sf9 cells with these antibodies, a ring-like structure was observed, as with Leu3a-PE. However, the fluorescence intensity was much weaker and appeared to be much more patchy. As shown in the following the lateral mobility of CD4 was large when Leu3a-PE was employed as fluorescent marker but much smaller when employing the FITC-labelled anti-CD4 antibodies. We assume (see the Discussion section) that the large PE moiety interferes sterically with one of the binding sites of Leu3a, thus rendering Leu3a-PE functionally monovalent. The specificity of the C6 antibody, raised against the intracellularly exposed C-terminus of full-length CD4, was also tested (Figures 4g–4j): on incubation of non-infected Sf9 cells or Sf9 cells expressing full-length CD4 with C6 and a rabbit anti-mouse IgG-FITC antibody, no fluorescent labelling could be detected.

Lateral mobility of CD4

The lateral mobility of the Leu3a-PE-labelled CD4 was measured by photobleaching (Figure 5). Each experimental curve had two phases: the fluorescence measurement before bleaching ($t = 0–20$ s) and after bleaching ($t = 20–170$ s). Mobility coefficients, i.e. the mobile fraction f_m and the lateral diffusion coefficient D , were derived by fitting the experimental data by the appropriate theoretical function (smooth line in Figure 5; note that although the fluorescence was measured every second, only every seventh data point is shown, for clarity). Labelling of CD4-expressing Sf9 cells with Leu3a-FITC, OKT4-FITC or OKT4A-FITC resulted in a very small mobility with $f_m = 22–28\%$ and $D = (2.6–3.3) \times 10^{-10} \text{ cm}^2/\text{s}$ (Figure 5; Table 1). This observation was not particularly surprising, because the labelling of membrane receptors with bivalent antibodies is well known to induce receptor immobilization by cross-linking and subsequent patching and even capping [36,37].

In contrast, Leu3a-PE-labelled full-length CD4 was found (Table 1) to have $D = (4.7 \pm 1.9) \times 10^{-10} \text{ cm}^2/\text{s}$ and $f_m = 80 \pm 16\%$.

The Leu3a-PE-labelled CD4 mutant was found to have $D = (5.7 \pm 2.3) \times 10^{-10} \text{ cm}^2/\text{s}$ and $f_m = 87 \pm 14\%$ (Table 2). Thus its lateral mobility was, within experimental error, the same as that of the full-length CD4 construct.

Effect of an anti-immunoglobulin antibody on the mobility of Leu3a-labelled CD4

When Leu3a-PE-labelled cells were incubated with a polyclonal goat anti-mouse anti-immunoglobulin antibody, the mobility of CD4 was strongly reduced (Figure 6; Table 2). This was true for both full-length (Figure 6a) and truncated CD4 (Figure 6b). In

Figure 4 Specificity of the anti-CD4 antibodies Leu3a-PE and C6

Sf9 cells were treated with antibodies and observed by phase-contrast (**a,c,e,g,i**) and immunofluorescence microscopy (**b,d,f,h,j**). (**a,b**) Sf9 cells expressing full-length CD4, labelled with Leu3a-PE. (**c,d**) Sf9 cells expressing truncated CD4, labelled with Leu3a-PE. (**e,f**) Non-infected Sf9 cells, labelled with Leu3a-PE. (**g,h**) Non-infected Sf9 cells, labelled with C6 and then with an FITC-conjugated rabbit anti-mouse IgG. (**i,j**) Sf9 cells expressing full-length CD4, labelled with C6 and then with an FITC-conjugated rabbit anti-mouse IgG. Scale bar, 20 μ m.

both cases the mobile fraction amounted to about 25%. The diffusion coefficients seemed to increase somewhat, but the deviations of these values are particularly large owing to the small mobile fraction. The decrease in mobility indicates that Leu3a-PE-labelled CD4 was cross-linked by the secondary antibody, similarly to the effect of FITC-conjugated primary antibodies on CD4 mobility.

Effect of intracellular application of the anti-CD4 antibody C6 on CD4 mobility

When C6 was microinjected into Leu3a-PE-labelled Sf9 cells expressing full-length CD4, f_m was reduced from $80 \pm 16\%$ to $42 \pm 17\%$ (Figure 6a; Table 2). A similar f_m was measured when C6 was introduced into cells by fusion with C6-loaded liposomes

Figure 5 Lateral mobility of full-length CD4 in the cell membrane of Sf9 cells labelled with various commercial monoclonal fluorescent anti-CD4 antibodies

Lateral mobility was measured by the photobleaching method. Representative experiments are shown, plotting in each case for clarity only 25 out of 170 measured values. Solid curves represent the best fit of data by the appropriate theoretical function. Symbols: ●, Leu3a-PE; ▲, Leu3a-FITC; ■, OKT4-FITC; ◆, OKT4A-FITC.

Table 1 Lateral mobility of full-length CD4 in the plasma membrane of Sf9 insect cells

Full-length CD4 was expressed in Sf9 insect cells. Plasma-membrane-inserted CD4 was labelled by incubating intact Sf9 cells with the different commercial monoclonal fluorescent anti-CD4 antibodies listed. The lateral mobility of labelled CD4 in the plasma membrane was measured by the fluorescence photobleaching technique to determine the mobile fraction f_m and the lateral diffusion coefficient D , each given as mean $\pm$ S.D. of n measurements.

Antibody	Fluorescent Group	f_m (%)	$D \times 10^{10}$ (cm ² /s)	n
Leu3a	PE	80 $\pm$ 16	4.7 $\pm$ 1.9	102
Leu3a	FITC	22 $\pm$ 15	2.9 $\pm$ 3.0	16
OKT4	FITC	26 $\pm$ 14	3.3 $\pm$ 3.9	17
OKT4A	FITC	28 $\pm$ 15	2.6 $\pm$ 3.7	16

Figure 6 Modulation of CD4 lateral mobility by antibodies

(a) Full-length CD4; (b) the truncated CD4 mutant. In all cases, CD4 or the CD4 mutant was labelled by Leu3a-PE and the lateral mobility measured by the photobleaching method. Representative experiments are shown, plotting in each case for clarity only 25 out of 170 measured values. Solid curves represent the best fit of data by the appropriate theoretical function. Symbols: ●, only Leu3a-PE antibody labelling; ▲, after microinjection of an antibody (13.B.8.2) of irrelevant specificity; ■, after microinjection of antibody C6; ◆, after incubation of cells with a secondary (goat anti-mouse) antibody.

(Table 2). No decrease in mobility was observed, however, when C6 was introduced into Sf9 cells expressing truncated CD4, independently of the delivery method employed (Figure 6b; Table 2). Furthermore, the intracellular delivery of control antibodies of irrelevant specificity (13.B.8.2, IgG_{2b}), employing either microinjection or fusion with liposomes, did not affect the

Table 2 Effect of antibodies on the lateral mobility of Leu3a-PE labelled CD4

Full-length (a) or truncated (b) CD4 was expressed in Sf9 insect cells. Plasma-membrane-inserted CD4 was fluorescently labelled by incubating intact Sf9 cells with the anti-CD4 antibody Leu3a-PE, generated in mouse. Then cells were treated in different ways with different antibodies and the effect of these treatments on the lateral mobility of CD4 was measured by the fluorescence photobleaching method (f_m , mobile fraction; D , lateral diffusion coefficient, each given as mean $\pm$ S.D. of n measurements).

			Lateral mobility		
Name	Antibody added (directed against)	Method of application	f_m (%)	$D \times 10^{10}$ (cm ² /s)	n
(a) Full-length CD4:					
None			80 ± 16	4.7 ± 1.9	102
Goat anti-mouse	Leu3a-PE	Addition to medium	25 ± 11	10.1 ± 4.9	27
C6	C-terminus of full-length CD4	Microinjection	42 ± 17	8.5 ± 6.1	33
13.B.8.2	Extracellular domain of CD4	Microinjection	70 ± 17	7.2 ± 4.1	23
C6	C-terminus of full-length CD4	Intracellular delivery by liposomes	44 ± 12	2.4 ± 1.6	9
IgG _{2b}	No anti-CD4 specificity	Intracellular delivery by liposomes	76 ± 14	5.3 ± 1.4	5
(b) Truncated CD4:					
None			87 ± 14	5.7 ± 2.3	58
Goat anti-mouse	Leu3a-PE	Addition to medium	23 ± 13	7.1 ± 2.9	27
C6	C-terminus of full-length CD4	Microinjection	79 ± 12	9.8 ± 4.3	33
13.B.8.2	Extracellular domain of CD4	Microinjection	74 ± 16	5.5 ± 2.8	19
C6	C-terminus of full-length CD4	Intracellular delivery by liposomes	76 ± 19	2.1 ± 1.2	6
IgG _{2b}	No anti-CD4 specificity	Intracellular delivery by liposomes	84 ± 15	3.4 ± 1.2	6

Figure 7 Effect of the phorbol ester PMA, the PKC inhibitor H7 and the calmodulin inhibitor W7 on the lateral mobility of full-length CD4 and the truncated CD4 mutant

CD4 was labelled with Leu3a-PE and mobility measured by the photobleaching method. Representative experiments are shown, plotting in each case for clarity only 25 out of 170 measured values. Solid curves represent the best fit of data by the appropriate theoretical function. (a) ●, Truncated CD4 after incubation with 100 nM PMA; ▲, full-length CD4 after incubation with 100 nM PMA; ■, truncated CD4 without PMA; ◆, full-length CD4 without PMA. (b) ●, Full-length CD4 after incubation with 25 μ M W7 and 100 nM PMA; ▲, full-length CD4 after incubation with 25 μ M H7 and 100 nM PMA; ■, full-length CD4 after incubation with 25 μ M H7, without PMA; ◆, full-length CD4 after incubation with 25 μ M W7, without PMA.

lateral mobility of full-length or truncated CD4 (Figure 6; Table 2). The diffusion coefficients of both CD4-Leu3a-PE constructs seemed to be somewhat enhanced after microinjection and diminished after delivery of C6 antibodies by cationic liposomes. However, the deviations are large and the differences might be due to altered lipid composition after fusion and by an expanded cytoplasm after microinjection.

Effects of PMA, a protein kinase C inhibitor and a calmodulin inhibitor on CD4 mobility

In mammalian cells, endocytosis of CD4 is initiated by clustering of CD4 and its entry into coated pits. The signalling pathway for these processes seems to comprise the PKC-mediated phosphorylation of CD4 residues Ser⁴⁰⁸ and Ser⁴¹⁵ [23,24] and/or a calmodulin/calcium-dependent way [38]. In lymphocytes, PMA stimulates phosphorylation and endocytosis of CD4, whereas the PKC inhibitor H7 blocks PKC-mediated phosphorylation of CD4, but not necessarily endocytosis. Endocytosis can be blocked, however, by the calmodulin inhibitor W7 [38]. The effects of PMA, H7 and W7 on the lateral mobility of CD4 are shown in Figure 7 and Table 3. Treatment with PMA decreased the mobile fraction of full-length CD4 to $53 \pm 10\%$, whereas the mobile fraction of truncated CD4 was not affected. Similar effects were observed when H7 treatment was followed by PMA treatment. However, when W7 treatment was followed by a PMA treatment no decrease in mobility was seen.

Table 3 Effect of the phorbol ester PMA, the protein kinase C inhibitor H7 and the calmodulin inhibitor W7 on CD4 lateral mobility

Full-length (a) or truncated (b) CD4 was expressed in Sf9 insect cells. Plasma-membrane-inserted CD4 was fluorescently labelled with Leu3a-PE. Then cells were treated with PMA, H7 and/or W7 as described in Materials and Methods, and the effect of these treatments on the lateral mobility of CD4 was measured by the fluorescence photobleaching method (f_m , mobile fraction; D , lateral diffusion coefficient, each given as mean $\pm$ S.D. of n measurements).

Treatment			Lateral mobility		
PMA	H7	W7	$f_m(\%)$	$D \times 10^{10} (\text{cm}^2/\text{s})$	n
(a) Full-length CD4:					
—	—	—	80 ± 16	4.7 ± 1.9	102
—	+	—	86 ± 13	4.0 ± 1.7	10
—	—	+	79 ± 13	4.1 ± 2.0	14
+	—	—	53 ± 10	3.8 ± 1.9	17
(b) Truncated CD4:					
—	—	—	87 ± 14	5.7 ± 2.3	58
+	—	—	86 ± 17	4.9 ± 2.0	16

DISCUSSION

In the present study the lateral mobility of full-length CD4 and a C-terminally truncated CD4 mutant was measured in the plasma membrane of living Sf9 insect cells by combining molecular genetic with immunological, cell biological and biophysical methods. The most essential results of the study are: (i) that CD4 could be fluorescently labelled by Leu3a-PE, a commercial antibody, without interfering with CD4 lateral mobility, (ii) that the mobility of Leu3a-PE-labelled CD4 was quite large, (iii) that the truncated CD4 had the same mobility as full-length CD4, and, most importantly, (iv) that an antibody with specificity for an intracellularly exposed CD4 domain of full-length CD4 could interact with full-length CD4 in the living cell and apparently cause a cross-linking of CD4 on the cytoplasmic membrane face.

Leu3a-PE-labelled full-length CD4 was found (Table 1) to have a lateral diffusion coefficient of $D = (4.7 \pm 1.9) \times 10^{-10} \text{ cm}^2/\text{s}$ and a mobile fraction of $f_m = 80 \pm 16\%$. Although a D of about $5 \times 10^{-10} \text{ cm}^2/\text{s}$ is average for integral membrane proteins [21,39,40], it is astonishing in the present context. We are dealing with the complex of an integral membrane protein and a bulky ligand, the Leu3a-PE moiety, which has a combined molecular mass of $\sim 300 \text{ kDa}$. That the diffusion coefficient was nevertheless quite large indicates that the resistance to lateral diffusion was due mainly to the lipid bilayer of the plasma membrane and the proteins in it and only to a minor, perhaps negligible, degree to the aqueous phase adjacent to the plasma membrane. Even more unexpected than the behaviour of the diffusion coefficient was the magnitude of the mobile fraction. In fact, Leu3a-PE behaved as though it were monovalent in a functional sense. We therefore assume that the large PE moiety interferes sterically with one of the binding sites of Leu3a. This assumption was supported by epitope calibration measurements with immunobeads (results not shown). Altogether, the results suggested that Leu3a-PE is an exceptional fluorescent label that faithfully reports on the lateral mobility of CD4.

The lateral mobility of truncated CD4 was, within experimental error, the same as that of the full-length CD4 construct. Full-length and truncated CD4 differed only by 32 C-terminal, cytoplasmically exposed, residues. This is a relatively small difference with regard to molecular mass and size and cannot be expected to cause a measurable difference in lateral mobility [41].

Differences in mobility could be expected only if the C-terminus of CD4 strongly interacted with rigid cellular structures such as the cytoskeleton. The absence of mobility differences therefore suggests that the C-terminus of CD4 did not interact with the cytoskeleton. Of course, this conclusion applies only to the Sf9 cells and not necessarily to lymphocytes, for example.

Previously, the lateral mobility of CD4 seems to have been the subject of only a single study [42], in which the HIV-protein gp120 was fluorescently labelled with FITC and then incubated with lymphocytes. FM measurements on the gp120-CD4 complex in lymphocyte membranes yielded a diffusion coefficient of $\sim 5 \times 10^{-10} \text{ cm}^2/\text{s}$ and a mobile fraction of 61 %. Thus, despite large differences in labelling methods and cellular systems, the previous study [42] and the present one yielded essentially identical diffusion coefficients. That the mobile fraction was smaller in lymphocytes than in Sf9 cells is not surprising. In lymphocytes, CD4 interacts with various proteins such as the T-cell receptor and tyrosine kinases [43,44]. In addition, in lymphocytes CD4 may well interact in a direct or indirect manner with the cytoskeleton, which would also reduce the mobile fraction.

The modulation of plasma-membrane mobility in the living cell by cross-linking on the internal, cytoplasmic membrane face has not to our knowledge been achieved previously. In the present study, specific and non-specific antibodies were delivered into the cytoplasm of living cells by two independent methods, microinjection and fusion with cationic liposomes. Both methods yielded the same result. It may be mentioned that cationic liposomes have been used recently in gene transfer [45,46], but not for delivery of proteins or antibodies. A very recent study showed encapsulation of a protein in cationic liposomes but not its delivery into cells [47].

The newly generated monoclonal antibody C6, specific for the cytoplasmic domain of full-length CD4 (residues 400–412) but not for the truncated CD4 mutant, reduced the mobile fraction to about one-half that in non-treated cells or cells previously loaded with non-specific antibodies. This indicates that the intracellularly delivered C6 antibody had access to its epitope and that the cytoplasmic CD4 domain was not hidden in a complex of cytoplasmic proteins. Moreover, the observed reduction of the mobile fraction after C6 delivery implies that CD4 did occur, at least partly, as a dimer or higher aggregate. If CD4 had occurred only in the form of monomers the monoclonal C6 antibody would have been able to generate CD4 dimers. These would have had only a slightly smaller diffusion coefficient than monomeric CD4, but not a smaller mobile fraction.

The phorbol ester PMA is known to induce endocytosis of CD4 in lymphoid and non-lymphoid cells. The mechanism of this process seems to be very complex and is essentially unknown. It has been suggested that endocytosis may be triggered by phosphorylation of CD4 residues Ser⁴⁰⁸ and Ser⁴¹⁵ [23]. This is questionable, however, because mutation of wild-type CD4 at Met⁴⁰⁷, Leu⁴¹³ or Leu⁴¹⁴ effectively blocks endocytosis but is nevertheless accompanied by an enhanced phosphorylation of Ser⁴⁰⁸ and Ser⁴¹⁵ [24]. Furthermore, inhibition of PKC in lymphocytes by H7 after PMA treatment inhibits CD4 phosphorylation but not endocytosis. Only the inhibition of a calmodulin/calcium-dependent pathway by the inhibitor W7 could block endocytosis in lymphocytes [38]. Therefore it appears more likely that phosphorylation of CD4 residues Ser⁴⁰⁸ and Ser⁴¹⁵ does occur during endocytosis but not as the primary signal. In our experiments the mobility of full-length but not truncated CD4 was affected by phorbol ester treatment, and this effect was abolished by a calmodulin inhibitor but not a PKC inhibitor. It has been shown in the meantime [48] that phorbol

ester in fact induces the endocytosis of CD4 and that endocytosis can be blocked by intracellular delivery of C6.

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