Intracellular Mediators Regulate CD2 Lateral Diffusion and Cytoplasmic Ca^{2+} Mobilization upon CD2-mediated T Cell Activation

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ABSTRACT CD2 is a T cell surface glycoprotein that participates in T cell adhesion and activation. These processes are dynamically interrelated, in that T cell activation regulates the strength of CD2-mediated T cell adhesion. The lateral redistribution of CD2 and its ligand CD58 (LFA-3) in T cell and target membranes, respectively, has also been shown to affect cellular adhesion strength. We have used the fluorescence photobleaching recovery technique to measure the lateral mobility of CD2 in plasma membranes of resting and activated Jurkat T leukemia cells. CD2-mediated T cell activation caused lateral immobilization of 90% of cell surface CD2 molecules. Depleting cells of cytoplasmic Ca^{2+}, loading cells with dibutyric cAMP, and disrupting cellular microfilaments each partially reversed the effect of CD2-mediated activation on the lateral mobility of CD2. These intracellular mediators apparently influence the same signal transduction pathways, because the effects of the mediators on CD2 lateral mobility were not additive. In separate experiments, activation-associated cytoplasmic Ca^{2+} mobilization was found to require microfilament integrity and to be negatively regulated by cAMP. By directly or indirectly controlling CD2 lateral diffusion and cell surface distribution, cytoplasmic Ca^{2+} mobilization may have an important regulatory role in CD2 mediated T cell adhesion.

INTRODUCTION

Cellular adhesion is an important event in many biological processes including immune cell responses (Springer, 1990). T lymphocyte adhesion to target membranes is mediated by several receptor-ligand pairs of cell surface molecules. CD2 and CD11a/CD18 (lymphocyte function-associated antigen 1 (LFA-1)) provide the T cell with two major adhesion pathways. CD2 binds CD58 (LFA-3), CD59 (Deckert et al., 1992; Hahn et al., 1992a), and CD48 (Kato et al., 1992; Arulanandam et al., 1993), whereas CD11a/CD18 binds CD54, CD102, and CD50 (intercellular adhesion molecules 1, 2, and 3, respectively) (Springer, 1990).

Cellular adhesion events are associated with redistribution of adhesion molecules to sites of contact between lymphocyte and target membranes (Ferguson et al., 1991; Koyasu et al., 1990). The strength of adhesion appears to be determined not only by the affinity of binding between receptors and ligands but also by the density of these adhesion molecules at the contact sites. Redistribution of adhesion molecules to contact sites requires that these molecules are able to diffuse laterally in the plasma membrane. The influence of the lateral mobility of adhesion molecules on the strength of cellular adhesion has been demonstrated by experiments in which Jurkat cells were found to adhere more strongly to laterally mobile CD58 than to immobile CD58 reconstituted in glass-supported planar bilayer membranes (P.-Y. Chan et al., 1991).

Activation of T cells initiates several intracellular signaling pathways that lead both to nuclear activation and to changes in the strength of T cell adhesion (Springer, 1990). Intracellular signals appear to regulate the avidity of adhesion mediated by receptor-ligand molecule pairs including CD11a/CD18:CD54 (Dustin and Springer, 1989; Van Kooyk et al., 1989), CD2:CD58 (Hahn et al., 1992b), CD8:MHC class I (O'Rourke et al., 1990), and VLA integrins (CD49d/CD29, CD49e/CD29):extracellular matrix proteins (Shimizu et al., 1990; B. M. Chan et al., 1991). In such interactions, intracellular signals could modulate the binding affinity and/or the lateral mobility of the adhesion molecules. Consistent with this hypothesis, correlations between cell activation and decreased membrane receptor lateral mobility have been observed in neutrophils stimulated with fMet-Leu-Phe (Johansson et al., 1993) and in the human T cell line HPB-ALL stimulated with bivalent anti-CD3 monoclonal antibody (mAb) (Hashemi et al., 1992).

Based on these observations, we hypothesized that intracellular signals regulate the lateral mobility of cell surface adhesion molecules. We examined the correlation between T cell activation and changes in the lateral mobility of the adhesion molecule CD2, and the mechanisms by which intracellular signals regulate CD2 mobility. In addition to its receptor function in cellular adhesion, the CD2 molecule itself can be stimulated to induce T cell activation. T cell proliferation and IL-2 production are triggered not only via the T cell receptor for antigen (TCR) but also upon binding

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Abbreviations used: ABP, actin binding proteins; $[Ca^{2+}]_i$, cytoplasmic free calcium ion concentration; *D*, lateral diffusion coefficient; Db cAMP, dibutyric cAMP; *f*, fractional mobility; FITC, fluorescein isothiocyanate; FPR, fluorescence photobleaching recovery; HBS, HEPES-buffered saline; LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; PIP_2, phosphatidylinositol-4,5-bisphosphate; SER, sheep erythrocyte rosetting; TCR, T cell receptor.

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of two CD2-specific signals including CD58 and an anti-CD2 mAb or pairs of anti-CD2 mAbs (Meuer et al., 1984; Brottier et al., 1985; Bernard et al., 1986; Bierer et al., 1988b). Also, T cell proliferation and IL-2 production are enhanced upon co-stimulation of the TCR and CD2 (Hahn et al., 1992a). Using the $CD2^+$ T leukemia cell line Jurkat, we examined first the effect of T cell activation on the lateral mobility of CD2. Activating pairs of anti-CD2 mAbs markedly decreased the laterally mobile fraction of CD2 molecules. We then used this activation-associated CD2 immobilization as a model system to study the roles of cytoplasmic mediators, including cytoplasmic free calcium ions ($[Ca^{2+}]_i$) and cAMP, and of microfilaments in modulating CD2 lateral diffusion.

MATERIALS AND METHODS

Cell culture

Jurkat T leukemia cells were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc., Logan, UT), 100 units/ml penicillin-100 μg/ml streptomycin (Sigma), 3 mM glutamine (Gibco BRL, Gaithersburg, MD) and 50 μM 2-mercaptoethanol (Sigma) at 37°C in a 5% CO_2 atmosphere.

mAbs

Murine anti-CD2 mAbs included TS2/18 (IgG1) (Sanchez-Madrid et al., 1982) and 9.6 (IgG2a) (Kamoun et al., 1981), and the murine anti-CD2R mAb was 9-1 (IgG3) (Bernard et al., 1986). mAbs 9.6 and 9-1 were the generous gifts of Drs. Paul Martin and Patrick Trown, respectively. TS2/18 and 9-1 were used as purified IgG; 9.6 was used as ascites fluid. Fab fragments of TS2/18 were prepared by papain treatment (Harlow, 1988). Immobilized papain (Calbiochem, San Diego, CA; 2 mg papain/ml gel) was activated by incubation in 100 mM cysteine, 100 mM Na acetate, 3 mM EDTA, pH 5.5 at 37°C for 30 min. Activated papain was washed 3 times with 100 mM Na acetate, 3 mM EDTA, pH 5.5. TS2/18 was incubated with activated papain beads at 37°C for 6 h and then dialyzed overnight against PBS, pH 7.5. Fc fragments and remaining intact antibody molecules were removed by incubation with affigel protein A (Bio-Rad Laboratories, Richmond, CA) at 4°C for 45 min. SDS-PAGE analysis of the fraction containing Fab fragments showed a single protein band at molecular weight 50,000 under nonreducing conditions. TS2/18 was conjugated to fluorescein isothiocyanate (FITC), as described (Golding, 1976; Stolpen et al., 1988). The protein concentration was determined by measuring the optical density at 280 nm. The FITC:mAb molar ratio was about 1, as determined by measuring the optical density at 493 and 280 nm (Stolpen et al., 1988). mAbs were centrifuged at 100,000 × *g* (Airfuge; Beckman Instruments, Inc., Palo Alto, CA) for 30 min or 16,000 × *g* (Eppendorf microcentrifuge; Brinkmann Instruments, Inc., Westbury, NY) for 1 h. Both conditions of centrifugation yielded equivalent experimental results (data not shown).

Treatment and labeling of cells with mAbs

Cells were washed with HEPES-buffered saline (HBS; 140 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM HEPES, 5.6 mM glucose, pH 7.4), incubated at 5×10^6 cells/ml with mAbs (50 μg/ml) on ice in the dark for 30 min, and washed with HBS.

Treatment of cells with mediators and cytoskeletal inhibitors

Cytochalasin D (Sigma) and ionomycin (Sigma) were dissolved in ethanol to make stock solutions of 1 mM and 250 μM, respectively. Other reagents were dissolved in HBS. All reagents were present in the cell suspension throughout the experiment.

Depletion of intracellular Ca^{2+}

Cells (5×10^6/ml) were incubated at room temperature with 10 mM EGTA (Sigma) and 10 μM ionomycin for 1 h. EGTA was used to chelate extracellular Ca^{2+} and ionomycin to increase plasma membrane Ca^{2+} permeability. Ca^{2+} efflux, driven by the outward $[Ca^{2+}]$ gradient across the membrane, thereby depleted intracellular Ca^{2+}. As a control, the extracellular medium alone was depleted of Ca^{2+} by incubation of cells with 10 mM EGTA for 2–5 min.

Loading of cells with dibutyric cAMP

Cells (5×10^6/ml) were treated at room temperature with 2 mM dibutyric cAMP (Db cAMP) (Sigma) or 2 mM butyrate (Sigma) for 15 min. Butyrate was used as a control for the effects of the most abundant degradation product of Db cAMP.

Inhibition of cytoskeletal function

Cells (about 10^6/ml) were treated at 37°C for 2 h 20 min with 20 μM cytochalasin or 20 μM colchicine, or for 2 h 20 min or 4 h with 20 μM cytochalasin and 20 μM colchicine.

Conditions

Sequential treatment(s) of cells employed the same conditions as those used for individual treatments (see Table 2).

After treatments Jurkat cells were incubated on ice in the dark for 30 min with either FITC-TS2/18, the mAb combination FITC-TS2/18 + 9-1, the combination TS2/18 + FITC-9-1, or the combination TS2/18 + 9-1. Each mAb was added at a concentration of 50 μg/ml.

None of the treatments induced changes in cell morphology by phase contrast microscopy. Ethanol controls did not affect the lateral mobility of CD2 labeled with TS2/18 + 9-1 (data not shown).

Fluorescence photobleaching recovery

FPR experiments (Axelrod et al., 1976) were performed at room temperature using an ACAS 570 interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI). The Gaussian beam radius was 1 μm, and excitation and emission wavelengths were 488 and 510 ± 5 nm, respectively. Photobleaching power at the sample was about 0.5 mW, and the bleaching time was 40–50 ms. The photobleaching beam was positioned in central areas of cells, away from the peripheral ring staining of the plasma membrane. The fractional mobility (*f*; i.e., the fraction of FITC-labeled protein that was free to diffuse in the plane of the membrane), and the diffusion coefficient (*D*) of the mobile fraction were obtained by using nonlinear least-squares analysis of fluorescence recovery curves (Golan et al., 1986) (see Fig. 1).

Cytoplasmic free calcium ion concentration ($[Ca^{2+}]_i$).

Fluo-3 (Molecular Probes, Eugene, OR) was dissolved at 1 mM in dimethylformamide (Fisher, Pittsburgh, PA). Fluo-3 solution was mixed with Pluronic F-127 (Molecular Probes) dissolved in dimethylformamide at 2 g of Pluronic F-127 per mole of fluo-3. Cells (5×10^6/ml) were incubated at room temperature for 1 h with 10 μM fluo-3 in HBS and then washed 3 times with HBS. The ACAS 570 interactive laser cytometer was used to image individual fluo-3-loaded cells using an excitation wavelength of 488 nm and an emission wavelength of 510 ± 5 nm, and the integrated single cell fluorescence intensity was measured at room temperature. Calibration of fluo-3

fluorescence and calculation of $[Ca^{2+}]_i$ were based on the method described by Kao et al. (1989). The fluorescence intensity of intracellular fluo-3 saturated with Mn^{2+} was obtained by incubating fluo-3-loaded cells at room temperature with 10 μM ionomycin for 5 min and then with 2 mM $MnCl_2$ for 15 min. One percent Triton X-100 (Bio-Rad Laboratories) was added to fluo-3-loaded cells to obtain the background fluorescence. Control single cell fluorescence intensity was measured immediately after fluo-3 loading. The mAb pair TS2/18 + 9-1 (each at a concentration of 50 μg/ml) was then added to the cell suspension, and single cell images were taken. All measurements were taken between 2 and 60 min after mAb addition, and results were analyzed as a function of time (see Results). A different cell was imaged for each data point. The fluorescence intensity of control cells did not vary over the time course of the experiments (data not shown). R_a represents the fraction of cells with $[Ca^{2+}]_i$ greater than 332 nM, which was the mean (116.8 nM) + 2 × SD (107.6 nM) intracellular $[Ca^{2+}]$ for resting control cells (n = 92). P, the percentage of Jurkat cells with elevated $[Ca^{2+}]_i$ in the absence of TS2/18 + 9-1 treatment, was used to estimate the number of high $[Ca^{2+}]_i$ cells in the background. This background was subtracted from T, the total number of cells measured, and from H, the number of cells with elevated $[Ca^{2+}]_i$ after addition of TS2/18 + 9-1. That is, $R_a = (H - T \times P)/(T - T \times P)$. P was calculated independently for each experiment; its magnitude was 3–42% for control cells, 14–35% for Db cAMP-treated cells, 0% for EGTA + ionomycin-treated cells, and less than 20% for cytochalasin-, colchicine-, EGTA-, and butyrate-treated cells.

RESULTS

Activation of Jurkat T cells is associated with lateral immobilization of cell surface CD2

We used $CD2^+$ Jurkat T leukemia cells to investigate the regulation of CD2 lateral mobility by T cell activation. Cells were stimulated with pairs of anti-CD2 mAbs, including TS2/18 + 9-1, which induced a significant increase in $[Ca^{2+}]_i$ (see below), and 9.6 + 9-1, which stimulates not only increased $[Ca^{2+}]_i$ but also T cell proliferation and IL-2 production (Bernard et al., 1986; Yang et al., 1986; Hahn et al., 1991). The anti-CD2 mAbs TS2/18, 9.6, and 9-1 (an anti-CD2R mAb) bind to different epitopes of CD2 (Peterson and Seed, 1987; Bierer et al., 1988a). The epitopes to which TS2/18 and 9.6 bind are highly expressed on resting T cells, whereas the 9-1 epitope (CD2R) is weakly present on resting cells and highly expressed on activated cells (Yang et al., 1987). Binding of TS2/18 (see below) or 9.6 (Yang et al., 1986, 1987) to CD2 enhances the expression of the CD2 epitope to which 9-1 binds on T cells. In contrast to treatment with stimulating pairs of anti-CD2 mAbs, treatment with the single mAb TS2/18 or with the Ab pair TS2/18 + goat anti-mouse IgG2a (an irrelevant antibody) did not induce an increase in $[Ca^{2+}]_i$ (data not shown). This finding is consistent with the observation that single anti-CD2 mAbs are unable to stimulate T cells to proliferate or to induce $[Ca^{2+}]_i$ elevation (Wallace et al., 1987). We therefore used TS2/18-labeled cells as controls for cells treated with stimulating pairs of anti-CD2 mAbs.

The fractional mobility (f) of FITC-TS2/18-labeled CD2 in plasma membranes of resting cells was 70%, and the lateral diffusion coefficient (D) was 7.2×10^{-10} cm^2 s^{-1} (Fig.1, *A* and *B*; Table 1). These f and D values are typical for many transmembrane proteins in biological membranes. Addition of the anti-CD2R mAb 9-1 to FITC-TS2/18-labeled cells caused the fractional mobility of CD2 to decrease to 37% after 10 min of incubation and to less than 10% after 60 min of incubation (Fig. 1, *C* and *D*; Fig. 2 *A*).[1] Similar reductions in CD2 mobility were observed in cells treated with the mAb pairs TS2/18 + FITC-9-1 and 9.6 + FITC-9-1 (Table 1), but were not observed in cells treated with the mAb pair TS2/18 + goat anti-mouse IgG2a. Cells treated with the single mAb FITC-9-1 did not exhibit sufficient fluorescence signal to allow performance of a valid FPR experiment (see above). Thus, the fraction of laterally mobile CD2 molecules decreased significantly upon stimulation of Jurkat T cells with activating pairs of anti-CD2 mAbs.

Because an apparent decrease in fractional mobility could result from internalization of a labeled cell surface receptor (Thatte et al., 1994), we investigated the possibility that pairs of anti-CD2 mAbs induce CD2 internalization and, therefore, apparent CD2 immobilization. Internalization of fluorescently labeled CD2 would be expected to shift the fluorescence intensity distribution from the periphery ("rim stain") to the center of cells. The distribution of fluorescence intensity along the axis of laser scanning was similar for resting cells and for cells stimulated with pairs of anti-CD2 mAbs, however (Fig. 1, *A* and *C*). The fluorescence intensity at the perimeter of FITC-TS2/18-labeled cells was 2.5–3-fold greater than that in the center of cells (Fig. 1 *A*), and anti-CD2 mAb pairs did not significantly alter this distribution of cell surface fluorescence intensity for at least 60 min after mAb treatment (Fig. 1 *C*). In addition, the fluorescence intensity in the center of FITC-TS2/18-labeled cells remained constant for at least 90 min after addition of 9-1 (Fig. 2 *D* and data not shown). These results suggest that internalization of fluorescence was not induced by pairs of anti-CD2 mAbs (Thatte et al., 1994), and that CD2 internalization was not responsible for the marked decrease in CD2 mobility. Rather, these results show that >90% of CD2 molecules were immobilized at the cell surface upon activation of T cells by pairs of anti-CD2 mAbs.

Extensive cross-linking of cell surface receptors by pairs of mAbs directed against different epitopes on the receptors could result in lateral immobilization. To investigate this possibility, we attempted to label CD2 with a nonactivating pair of anti-CD2 mAbs, FITC-TS2/18 + 9.6. We found, however, that 9.6 markedly decreased the fluorescence intensity of FITC-TS2/18 on the cell surface, causing the signal to be too weak for performance of a valid FPR experiment.[2] As a second control, cells were simultaneously treated with FITC-9-1 and Fab fragments of TS2/18. This mAb combination, which is not capable of extensively cross-linking cell surface CD2 molecules, nonetheless induced both a significant increase in $[Ca^{2+}]_i$ (R_a = 0.43;

[1] After the 60 min incubation period, >90% of both control and activated cells remained viable as measured by trypan blue exclusion.

[2] Although TS2/18 and 9.6 have been found to bind to different epitopes of CD2 (Bierer et al., 1988a), steric hindrance interactions between TS2/18 and 9.6 may prevent simultaneous binding of these two anti-CD2 mAbs.

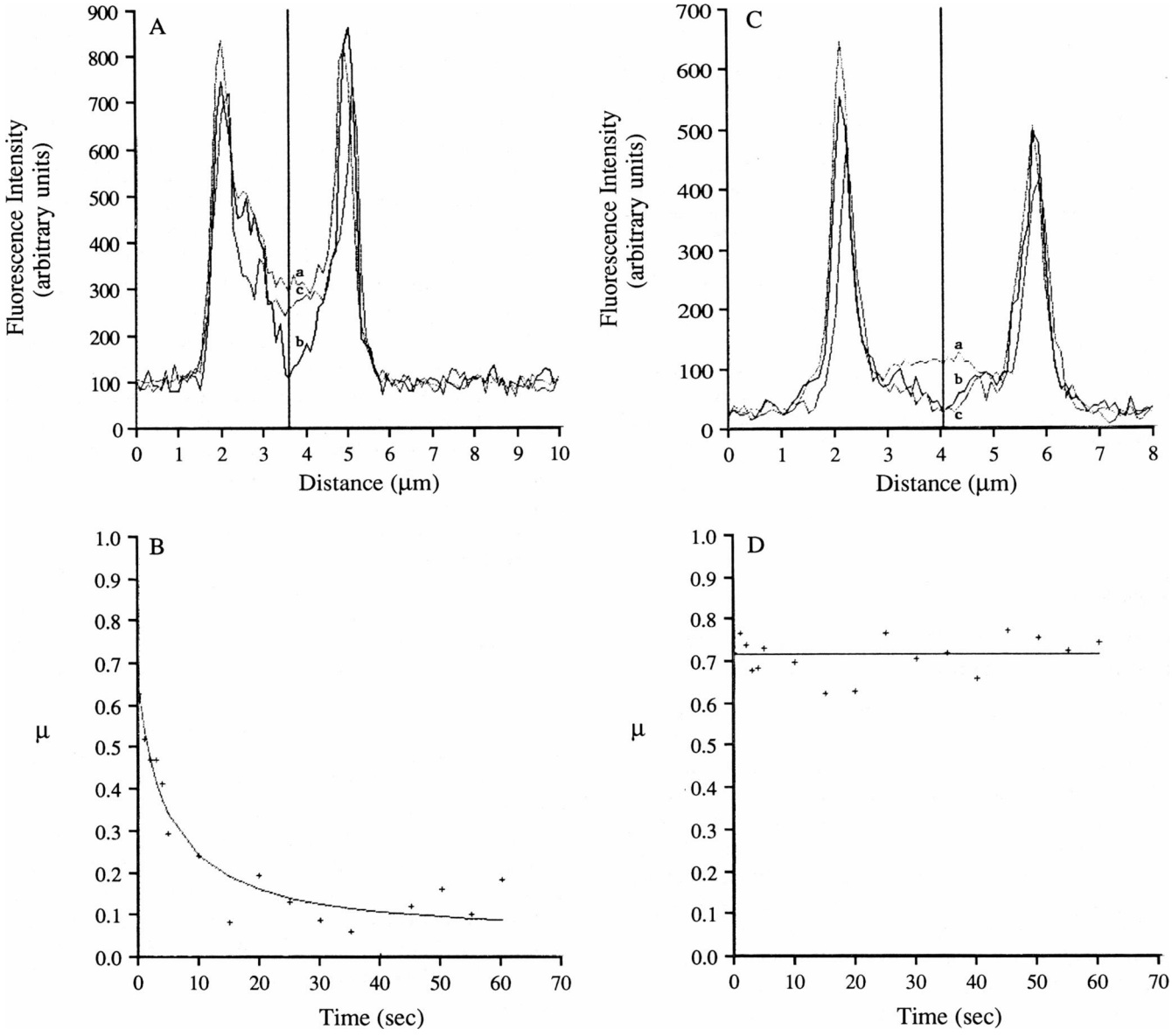

FIGURE 1 Representative fluorescence photobleaching recovery (FPR) measurements of CD2 lateral mobility in resting Jurkat T cells labeled with FITC-TS2/18 (*A*, *B*) and in activated cells labeled with the mAb combination FITC-TS2/18 + 9-1 (*C*, *D*). *A* and *C* show the linear profiles of fluorescence intensity before the photobleach (a), immediately after the photobleach (b), and 60 s after the photobleach (c) obtained by using the Meridian ACAS 570 interactive laser cytometer in line scan mode. The location of the photobleach is marked by a vertical line. The corresponding FPR curves are presented in *B* and *D*. Crosses represent experimental data points. μ is the fraction by which the fluorescence intensity is decreased after the photobleach at time = 0. Data points are fitted by nonlinear least-squares analysis. (*A*, *B*) $D = 6.1 \times 10^{-10}$ cm^2 s^{-1}; $f = 94\%$. (*C*, *D*) $f = 0\%$; *D* cannot be measured.

see legend to Fig. 3) and lateral immobilization of CD2 (Table 1). T cell activation, rather than cross-linking of CD2 molecules at the cell surface, therefore appeared to be responsible for the lateral immobilization of CD2 induced by pairs of anti-CD2 mAbs.

Cross-linking of CD2 by pairs of anti-CD2 mAbs could also cause clustering of CD2 into patches in the plasma membrane. Fluorescence imaging of single cells labeled with FITC-TS2/18 or FITC-TS2/18 + 9-1 showed peripheral ring staining of the plasma membrane for at least 60 min after mAb labeling. Twenty-nine percent of FITC-TS2/18-labeled (control) cells ($n = 37$) and 46% of FITC-TS2/18 + 9-1-treated (activated) cells ($n = 37$) were found to have 1–2 small (≤ 2 μm diameter) fluorescent patches in the membrane. Therefore, the presence of CD2 patches did not depend on treatment with stimulating pairs of anti-CD2 mAbs, although cell activation through CD2 modestly increased CD2 patching. The modest difference between the fractions of activated and control cells with FITC-TS2/18-labeled patches could not account for the profound decrease in CD2

TABLE 1 Pairs of anti-CD2 mAbs cause lateral immobilization of CD2 in Jurkat T cells

1st mAb	2nd mAb	D	f	N
FITC-TS2/18		7.2 ± 0.2*	70 ± 1 (1, 2, 3, 4)	262
TS2/18	FITC-9–1	ND	<10 (1)	47
FITC-TS2/18	9–1	ND	<10 (2)	102
9.6	FITC-9–1	ND	<10 (3)	48
TS2/18(Fab)	FITC-9–1	ND	13 ± 2 (4)	49

D, diffusion coefficient, $\times 10^{10}$ cm^2 s^{-1}; f, fractional mobility, %; N, number of measurements; ND, D cannot be determined for $f < 20\%$.
*Mean ± SEM.
1, 2, 3, 4: $p < 0.001$, Student two-tailed t-test.

lateral mobility, however. These results support the conclusion that excessive cross-linking of CD2 was not responsible for CD2 immobilization induced by pairs of anti-CD2 mAbs.

Roles of intracellular mediators in activation-associated CD2 immobilization

Intracellular signals induced by cell activation appear to regulate the avidity of adhesion molecules such as CD2 (Hahn et al., 1992b). We therefore examined whether activation-associated CD2 immobilization is mediated by intracellular signals. Because the stimulating mAb pair TS2/18 + 9-1 caused a significant increase in $[Ca^{2+}]_i$ (Fig. 2) whereas the single mAb TS2/18 did not elevate $[Ca^{2+}]_i$ (data not shown), we first investigated the role of cytoplasmic Ca^{2+} mobilization in CD2 immobilization.

Pretreatment of cells with EGTA and ionomycin was found to reduce $[Ca^{2+}]_i$ in resting cells and to prevent the $[Ca^{2+}]_i$ increase induced by treatment with TS2/18 + 9-1 (Fig. 3). Cells depleted of intracellular Ca^{2+} also manifested fewer patches of CD2 in the plasma membrane (14% of FITC-TS2/18-labeled (control) cells (n = 50) and 38% of FITC-TS2/18 + 9-1-treated (activated) cells (n = 50)). Depletion of $[Ca^{2+}]_i$ by EGTA + ionomycin partially reversed the lateral immobilization of CD2 induced by TS2/18 + 9-1, without significant effect on the mobility of CD2 in resting, FITC-TS2/18-labeled cells (Table 2). The latter observation, together with the finding that EGTA alone did not alter the mobility of CD2 labeled with FITC-TS2/18 (Table 2), renders unlikely the possibility that perturbation of membrane structure by ionomycin affected CD2 mobility directly. Depletion of extracellular Ca^{2+} by EGTA had little effect on

Figure 2

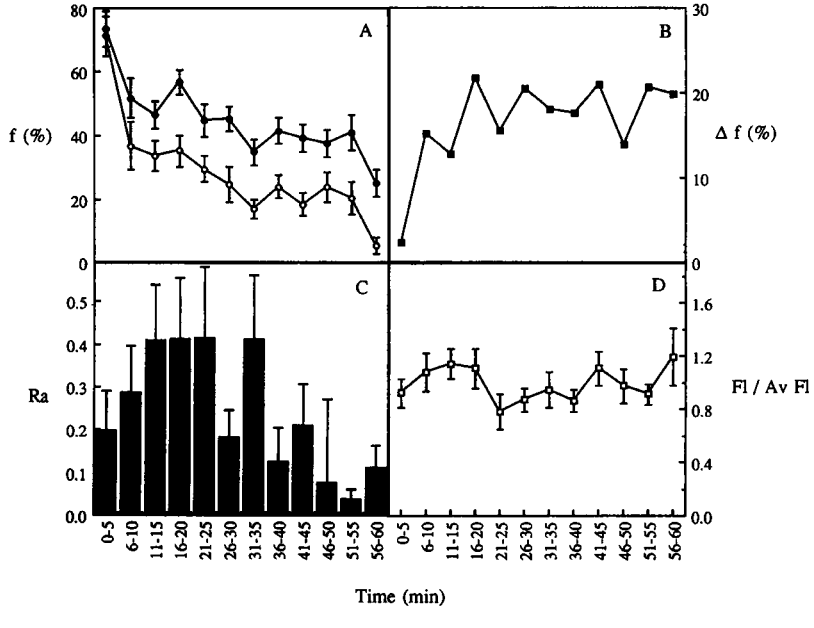

FIGURE 2 Kinetics of CD2 fractional mobility (*A*, *B*), intracellular $[Ca^{2+}]$ (*C*), and membrane fluorescein fluorescence intensity (*D*) in Jurkat T cells stimulated by the mAb combination FITC-TS2/18 + 9-1. (*A*) Cells were labeled with FITC-TS2/18 (50 μg/ml). mAb 9-1 (50 μg/ml) was then added to the cell suspension immediately before FPR measurements (○). In some experiments cells were pretreated with 10 mM EGTA and 10 μM ionomycin at room temperature for 1 h, labeled with FITC-TS2/18, and then treated with 9-1 (●). Data points represent mean ± SEM of 10–20 measurements from 6 independent experiments in each time interval. By the Student two-tailed t-test, $p > 0.1$ for f values with and without EGTA + ionomycin pretreatment between 0 and 10 min, and $p < 0.02$ for f values between 11 and 60 min after the addition of 9-1. (*B*) Difference, Δf, between the fractional mobility of CD2 with and without EGTA + ionomycin pretreatment. Primary data are shown in *A*. (*C*) Cells were loaded with fluo-3. TS2/18 (50 μg/ml) and 9-1 (50 μg/ml) were added to the cell suspension immediately before measurements of integrated cellular fluorescence intensity, which commenced at time = 0. R_a is the fraction of cells with $[Ca^{2+}]_i$ significantly greater than that of resting control cells, calculated as described in Materials and Methods. Data points represent mean ± SEM of 5 independent experiments, each of which involved $[Ca^{2+}]_i$ measurements on 15–47 cells in each time interval. (*D*) Cells were labeled with FITC-TS2/18 (50 μg/ml) and then with 9-1 (50 μg/ml). The fluorescence intensity in the center of each cell was measured as a function of time after the addition of 9-1, which occurred at time = 0. The fluorescence intensity (Fl) was then normalized by the average fluorescence intensity (Av Fl) for each individual experiment. Data points represent mean ± SEM of 15–19 measurements from 6 independent experiments in each time interval.

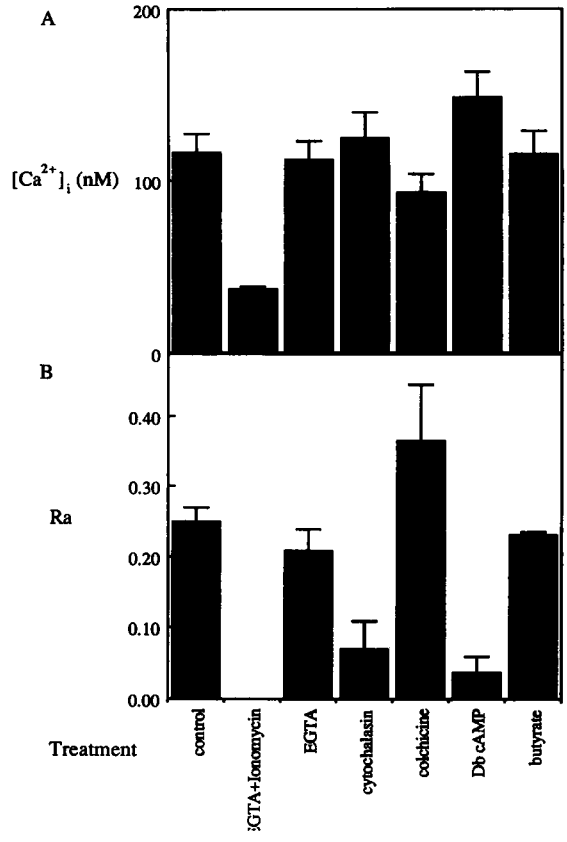

FIGURE 3 Effects of cellular pretreatments on $[Ca^{2+}]_i$ in resting Jurkat T cells (*A*) and on the elevation in $[Ca^{2+}]_i$ stimulated by treatment with TS2/18 + 9-1 (*B*). Intracellular Ca^{2+} concentration was calculated as described in Materials and Methods. EGTA + ionomycin, cytochalasin, and colchicine pretreatments were carried out before fluo-3 loading, and EGTA, Db cAMP, and butyrate pretreatments were performed after fluo-3 loading. (*A*) Data points represent mean ± SEM of measurements on 92 control, 50 EGTA + ionomycin-, 65 EGTA-, 62 cytochalasin-, 37 colchicine-, 51 Db cAMP-, and 60 butyrate-pretreated cells. By the Student two-tailed *t*-test, compared with the $[Ca^{2+}]_i$ value of control cells, $p > 0.2$ for the $[Ca^{2+}]_i$ values of EGTA-, cytochalasin-, colchicine-, and butyrate-pretreated cells, $p = 0.1$ for the $[Ca^{2+}]_i$ value of Db cAMP-pretreated cells, and $p < 0.001$ for the $[Ca^{2+}]_i$ value of EGTA + ionomycin-pretreated cells. (*B*) TS2/18 (50 μg/ml) and 9-1 (50 μg/ml) were added to fluo-3-loaded cells immediately before measurements of cellular fluorescence. R_a was calculated as described in Materials and Methods. R_a was determined over a period of 60 min after addition of TS2/18 + 9-1. The background, which was measured after each pretreatment, was subtracted from the experimental R_a value. Data points represent mean ± SEM of five independent experiments for control cells, three independent experiments for EGTA-, cytochalasin-, Db cAMP-, and butyrate-pretreated cells, and two independent experiments for EGTA + ionomycin- and colchicine-pretreated cells. By the Student two-tailed *t*-test, compared with the R_a value for control cells, $p < 0.01$ for the R_a value of EGTA + ionomycin, cytochalasin and Db cAMP pretreated cells, $p < 0.05$ for the R_a value of colchicine treated cells, and $p > 0.2$ for the R_a value of EGTA- and butyrate-pretreated cells. Data from all experiments were also pooled, and the aggregate R_a values were calculated for each pretreatment. The probabilistic independence of each pretreatment was tested by contingency table analysis (StatView 512$^+$, Brain Power, Inc., Calabasas, CA). *p* values compared to control were <0.0001 for EGTA + ionomycin, cytochalasin, and Db cAMP pretreatments, and >0.2 for EGTA, colchicine, and butyrate treatments. The total numbers of cells examined were 444 for control, 204 for EGTA + ionomycin, 315 for EGTA, 250 for cytochalasin, 185 for colchicine, 284 for Db cAMP, and 331 for butyrate pretreatments. R_a for cells activated with TS2/18(Fab) + 9-1 was 0.43, and the total number of cells examined was 77.

$[Ca^{2+}]_i$ in resting cells or on the $[Ca^{2+}]_i$ increase stimulated by pairs of anti-CD2 mAbs (Fig. 3). In contrast to the effect of EGTA + ionomycin, EGTA alone only minimally reversed the CD2 immobilization induced by TS2/18 + 9-1 (Table 2). These data suggest that low extracellular $[Ca^{2+}]$ was not primarily responsible for the reversal of CD2 immobilization by treatment with EGTA + ionomycin. Instead, the data imply that EGTA + ionomycin reversed activation-associated CD2 immobilization by affecting cytoplasmic Ca^{2+} mobilization in stimulated cells. It is also possible that treatment with EGTA + ionomycin perturbed the signal transduction machinery of the cell independent of an effect on intracellular $[Ca^{2+}]$. These results are consistent with our recent observation that cytoplasmic Ca^{2+} mobilization, stimulated by cross-linking of the TCR/CD3 complex, causes partial immobilization of CD2 in Jurkat T cell membranes (Liu and Golan, 1994a).

To examine further the relationship between increased $[Ca^{2+}]_i$ and CD2 immobilization, we compared the kinetics of mAb-induced changes in $[Ca^{2+}]_i$ and CD2 lateral mobility in control cells and in cells pretreated with EGTA + ionomycin (Fig. 2). In control cells $[Ca^{2+}]_i$ increased immediately upon stimulation with TS2/18 + 9-1, whereas CD2 lateral mobility decreased 10 min after stimulation. Therefore, intracellular Ca^{2+} mobilization cannot be a consequence of CD2 immobilization but it could serve to trigger CD2 immobilization. Both the difference (Δf) between the fractional mobility of CD2 with and without EGTA + ionomycin pretreatment and the level of increase in $[Ca^{2+}]_i$ in control cells increased over the first 20 min after mAb treatment (Fig. 2, *B* and *C*), suggesting that release of Ca^{2+} from intracellular stores is important for CD2 immobilization. $[Ca^{2+}]_i$ was found to decline about 35 min after mAb treatment, whereas Δf remained constant at a level of about 20% (Fig. 2, *B* and *C*). Together with the observed 10 min delay between the initial increase in $[Ca^{2+}]_i$ and the initial decrease in CD2 mobility, these data suggest that the modulation of CD2 mobility by $[Ca^{2+}]_i$ is mediated by intermediate molecular events. Consistent with this hypothesis, our recent finding that calmodulin inhibition partially reverses the CD2 immobilization induced by pairs of anti-CD2 mAbs (Liu and Golan, 1994b) suggests that calmodulin serves as one mediator of Ca^{2+}-associated CD2 immobilization. We attempted to increase cytoplasmic $[Ca^{2+}]$ by treatment with ionomycin and 1 mM extracellular Ca^{2+}, and thereby to measure directly the effect of increased $[Ca^{2+}]_i$ on CD2 lateral mobility. We found, however, that both cytoplasmic Ca^{2+} concentrations and CD2 fractional mobilities varied significantly among cells after ionomycin + Ca^{2+} treatment. For example, *f* values ranged from <10 to 70%, with an average value of 27 ± 6% ($n = 19$). Also, sustained high $[Ca^{2+}]_i$ typically resulted in cell death.

cAMP up-regulates the binding of CD2$^+$ cell lines to purified CD58 (Hahn et al., 1993), and binding of CD58 or TS2/18 to CD2 transiently increases cAMP levels in both T cell lines and resting purified T cells (Hahn et al., 1991). To examine the effect of cAMP levels on CD2 mobility, we

TABLE 2 Intracellular Ca^{2+}, cAMP, and microfilaments regulate CD2 lateral mobility in membranes of activated Jurkat T cells

	mAb Labeling					
	FITC-TS2/18			TS2/18+9–1*		
Treatment	*D*	*f*	*N*	*D*	*f*	*N*
None	7.2 ± 0.2‡	70 ± 1	262	ND	<10 (1, 2, 3)	149
a) EGTA+Ionomycin	8.8 ± 0.5	67 ± 2	77	7.5 ± 0.6	35 ± 3 (1, 4)	52
b) EGTA	7.5 ± 0.4	73 ± 2	65	ND	16 ± 2 (4)	80
c) Db cAMP	7.8 ± 0.4	59 ± 2	140	7.2 ± 0.7	29 ± 3 (2)	63
d) Butyrate	6.0 ± 0.5	50 ± 4	37	ND	11 ± 3	17
e) Cytochalasin	7.9 ± 0.6	68 ± 2	41	7.8 ± 0.7	30 ± 2 (3)	49
f) Colchicine	6.5 ± 0.7	71 ± 3	25	ND	<10	45
g) e+f, 2 hr 20 min	8.4 ± 0.8	73 ± 5	10	8.8 ± 1.3	25 ± 4	21
h) e+f, 4 hr				9.8 ± 2.2	25 ± 3	10
a → c	7.7 ± 1.2	62 ± 5	21	7.2 ± 1.6	38 ± 4	26
a → e	8.9 ± 0.7	75 ± 3	38	6.2 ± 0.7	40 ± 3	36
c → e	6.7 ± 0.3	74 ± 2	44	7.9 ± 0.9	31 ± 2	65
a → c → e	9.9 ± 1.9	71 ± 6	17	7.0 ± 1.2	41 ± 4	20

D, diffusion coefficient, $\times 10^{10}$ cm^2 s^{-1}; *f*, fractional mobility, %; *N*, number of measurements; ND, *D* cannot be determined for $f < 20\%$.
*Pairs of mAbs, including both FITC-TS2/18 + 9–1 and TS2/18 + FITC-9–1.
‡Mean ± SEM.
1,2,3,4: $p < 0.001$; Student two-tailed *t*-test.

loaded cells with the cAMP analog dibutyric cAMP (Db cAMP). Like EGTA + ionomycin pretreatment, Db cAMP loading partially reversed the immobilization of CD2 induced by TS2/18 + 9-1. In contrast, the functionally inactive metabolite of Db cAMP, butyrate, had no effect on mAb-induced CD2 immobilization (Table 2). The slight reduction in CD2 fractional mobility in resting cells treated with Db cAMP could be due to the effect of butyrate released as a breakdown product of intracellular Db cAMP, because butyrate alone reduced the *f* value of CD2 in resting cells (Table 2). Because pretreatment with Db cAMP increased CD2 fractional mobility in cells stimulated with pairs of anti-CD2 mAbs but not in resting cells, cAMP appears to interrupt the signal transduction pathway by which T cell activation leads to CD2 immobilization.

CD2 is the receptor on T cells that mediates rosetting of sheep erythrocytes (Van Wauwe et al., 1981; Kamoun et al., 1981; Howard et al., 1981). Erythrocyte rosette formation is prevented by pretreating T cells with the microfilament disrupting agent cytochalasin but not with the microtubule inhibitor colchicine (Freed et al., 1989; Ishijima et al., 1991). Further, CD2 appears to associate with α/β tubulin, an association that is decreased by CD2-mediated T cell activation (Offringa and Bierer, 1993). We therefore examined the roles of cytoskeletal proteins in regulating CD2 mobility by treating cells with cytochalasin or colchicine. These treatments did not affect the ability of fluorescent anti-CD2 mAbs to label CD2. Pretreatment of cells with cytochalasin caused the fractional mobility of CD2 in cells stimulated with FITC-TS2/18 + 9-1 to increase significantly, but had no effect on the mobility of CD2 in TS2/18-labeled resting cells (Table 2). These data suggest that microfilaments are involved in the regulation of CD2 mobility in activated cells but not in unstimulated cells. CD2 immobilization by TS2/18 + 9-1 could be at least partially mediated either by modulation of direct interactions between CD2 and the microfilament-based cytoskeleton or by an indirect microfilament-mediated signal transduction process. Pretreatment of cells with colchicine did not alter either the immobilization of CD2 by TS2/18 + 9-1 or the lateral mobility of CD2 in resting cells (Table 2). Therefore, microtubules do not appear to regulate CD2 mobility in either activated or unstimulated cells.

Having shown that perturbations of $[Ca^{2+}]_i$, cAMP levels and microfilament integrity individually modulate CD2 mobility, we investigated whether these intracellular mediators act through independent pathways. Combinations of these pretreatments followed by incubation with TS2/18 + 9-1 yielded CD2 fractional mobilities of 33–41% (Table 2), showing no additive effects. $[Ca^{2+}]_i$, cAMP, and microfilament integrity therefore appear to be involved in the same signal transduction pathway leading to mAb-induced CD2 immobilization.

Effects of intracellular mediators on activation-associated Ca^{2+} mobilization

Because increased $[Ca^{2+}]_i$ correlated directly with CD2 immobilization (Fig. 2, Table 1), we attempted to dissect the signal transduction pathways leading to CD2 immobilization by examining the effects of intracellular mediators on the increase in $[Ca^{2+}]_i$ induced by TS2/18 + 9-1 treatment (Fig.3). As expected, pretreatment of resting cells with EGTA + ionomycin significantly reduced $[Ca^{2+}]_i$ and abolished the $[Ca^{2+}]_i$ rise stimulated by TS2/18 + 9-1. Mobilization of cytoplasmic Ca^{2+} was predominantly responsible for the mAb-induced increase in $[Ca^{2+}]_i$, because pretreatment of cells with EGTA alone did not significantly reduce the $[Ca^{2+}]_i$ rise (Fig. 3). Cytochalasin pretreatment inhibited cytoplasmic Ca^{2+} mobilization, whereas colchicine pretreatment had no effect. Because treatment with cytochalasin did not alter $[Ca^{2+}]_i$ in resting cells, it is unlikely that cytochalasin regulates $[Ca^{2+}]_i$ directly. Rather, cytochalasin appears

to inhibit mAb-induced Ca^{2+} mobilization by disruption of cellular microfilaments. CD2-mediated T cell activation may therefore require microfilament integrity. Cells pretreated with Db cAMP, like those pretreated with cytochalasin, did not manifest an increase in $[Ca^{2+}]_i$ in response to TS2/18 + 9-1 treatment (Fig. 3). Pretreatment with butyrate, in contrast, did not inhibit Ca^{2+} mobilization upon mAb-induced cell activation. The specific effect of Db cAMP in our system is consistent with the observations that cAMP blocks the $[Ca^{2+}]_i$ increase induced by the anti-CD3 mAb OKT3 (Papadogiannakis et al., 1989) and reduces diacylglycerol and inositol phosphate generation stimulated by pairs of anti-CD2 mAbs (Bismuth et al., 1988).

DISCUSSION

We have shown that pairs of anti-CD2 mAbs markedly reduce CD2 lateral mobility in Jurkat T leukemia cells, and that this effect correlates with the induction of elevated $[Ca^{2+}]_i$. EGTA and ionomycin, Db cAMP, and cytochalasin treatments individually inhibit both the increase in $[Ca^{2+}]_i$ and the immobilization of CD2. Also, each of these treatments appears to act on a common pathway leading to CD2 immobilization. The ability of EGTA and ionomycin, Db cAMP, and cytochalasin to affect CD2 mobility appears to be mediated, at least in part, by inhibition of the increase in $[Ca^{2+}]_i$ that is stimulated in untreated cells by pairs of anti-CD2 mAbs. Finally, the inability of treatment combinations affecting $[Ca^{2+}]_i$-, cAMP-, and microfilament-mediated signal transduction to reverse fully the immobilization of CD2 associated with cell activation suggests that other, $[Ca^{2+}]_i$-independent signal transduction pathways are also involved in the regulation of CD2 mobility.

Several lines of evidence are consistent with the hypothesis that mobilization of cytoplasmic Ca^{2+} mediates lateral immobilization of CD2 at the cell surface. First, depletion of intracellular Ca^{2+} partially prevents CD2 immobilization by anti-CD2 mAb pairs. Second, loading cells with Db cAMP and disrupting microfilaments with cytochalasin are two other treatments that both inhibit cytoplasmic Ca^{2+} mobilization and reverse CD2 immobilization to the same degree as treatment with EGTA + ionomycin. Colchicine treatment does not affect either $[Ca^{2+}]_i$ or CD2 lateral mobility, and the effects of neither Db cAMP nor cytochalasin are additive. Third, cytoplasmic Ca^{2+} mobilization is temporally correlated with CD2 immobilization.

We show here that Jurkat T cell activation correlates with CD2 immobilization at the cell surface. Similar associations between cell activation and decreased membrane receptor mobility have been observed in neutrophils stimulated with fMet-Leu-Phe (Johansson et al., 1993) and in the human T cell line HPB-ALL stimulated with bivalent anti-CD3 mAb (Hashemi et al., 1992). In several different systems, then, it appears that either cell activation results in immobilization of surface receptors or cell stimulation requires receptor molecules to be immobilized. In both neutrophils and HPB-ALL cells, activation is associated with elevation of $[Ca^{2+}]_i$. Here we find that $[Ca^{2+}]_i$ increases immediately before CD2 immobilization. Increasing $[Ca^{2+}]_i$ could therefore serve to trigger CD2 immobilization. Considering pairs of anti-CD2 mAbs as a model for ligand-induced cell activation, we postulate that ligand binding to CD2 causes activation-associated release of intracellular Ca^{2+}, which in part mediates CD2 immobilization.

We have recently found that activation of Jurkat T cells by cross-linking the TCR/CD3 complex reduces CD2 fractional mobility from 70 to 50%, and that the partial immobilization of CD2 is completely reversed in cells depleted of $[Ca^{2+}]_i$ with EGTA + ionomycin (Liu and Golan, 1994a). In the present study, we show that depletion of intracellular $[Ca^{2+}]$ partially reverses the CD2 immobilization induced by pairs of anti-CD2 mAbs from an f value of 10% to 35%. The difference (Δf) between the fractional mobility of CD2 with and without EGTA + ionomycin treatment is therefore 20–25% in cells activated either by pairs of anti-CD2 mAbs or by cross-linking the TCR/CD3 complex. Thus, the effects of Ca^{2+} mobilization on CD2 lateral mobility are similar for cells activated through CD2 and TCR/CD3 pathways. We have also found that calmodulin inhibition partially prevents the CD2 immobilization induced by pairs of anti-CD2 mAbs and completely prevents the CD2 immobilization stimulated by cross-linking the TCR/CD3 complex (Liu and Golan, 1994b). These effects of calmodulin inhibition are consistent with those of EGTA + ionomycin treatment, and with the hypothesis that intermediate molecular events in the signal transduction pathway mediate Ca^{2+}-induced CD2 immobilization. Thus, activation-associated Ca^{2+} mobilization appears to initiate an intracellular signaling pathway that regulates CD2 lateral mobility in T cells, although Ca^{2+}-induced CD2 immobilization alone cannot account for the degree of CD2 immobilization stimulated by pairs of anti-CD2 mAbs.

Microfilaments are involved in a number of adhesive interactions including co-aggregation of W256 tumor cells and platelets (Chopra et al., 1988) and self-aggregation of mouse L cells transfected with liver cell adhesion molecules (Jaffe et al., 1990). The cytoplasmic domain of the integrin CD54, which together with CD2 mediates T cell adhesion, binds directly to α-actinin and the actin-containing cytoskeleton (Carpen et al., 1992). The observation that cytochalasin treatment decreases sheep erythrocyte rosetting (SER) suggests a possible role for microfilament integrity in T cell adhesion mediated by CD2 (Freed et al., 1989; Ishijima et al., 1991). We find here that cytochalasin treatment partially prevents CD2 immobilization by activating pairs of anti-CD2 mAbs. Because cytochalasin affects CD2 mobility on mAb-treated but not resting cells, microfilament integrity appears to be important in activation-associated CD2 immobilization but not in regulating CD2 mobility in resting cells.

In the present study, cells were treated with 20 μM cytochalasin, which is likely sufficient to disrupt cytosolic as well as membrane-associated microfilaments (Mookerjee et al.,

1981). The inhibitory effects of cytochalasin on mAb-induced $[Ca^{2+}]_i$ mobilization and CD2 lateral immobilization could therefore result from disruption of membrane-associated and/or cytosolic microfilaments. Because actin binding proteins (ABP) such as L-plastin and profilin play important roles in coordinating and regulating actin polymerization and the structure of the actin network (Forscher, 1989; Pollard and Cooper, 1986; Kabsch and Vandekerckhove, 1992; Namba et al., 1992; Pacaud and Derancourt, 1993), ABP could also mediate interactions between $[Ca^{2+}]_i$ and CD2. The abundant ABP L-plastin binds actin tightly and cross-links (bundles) actin efficiently at low concentrations of free calcium ions ($[Ca^{2+}] < 150$ nM); at higher calcium concentrations ($K_I = 1600$ nM), actin bundling is inhibited (Namba et al., 1992; Pacaud and Derancourt, 1993). ABP functions are regulated not only by Ca^{2+} but also by phosphatidylinositol-4,5-bisphosphate (PIP_2) (Forscher, 1989; Pollard and Cooper, 1986; Kabsch and Vandekerckhove, 1992; Lassing and Lindberg, 1985; Hartwig et al., 1989; Janmey and Stossel, 1989). Interactions between profilin and PIP_2 release actin monomers from profilin/actin complexes (Lassing and Lindberg, 1985) and protect PIP_2 from hydrolysis by phospholipase C (Goldschmidt-Clermont et al., 1990; 1991). ABP may therefore regulate polyphosphoinositide turnover as well as actin remodeling (Forscher, 1989). Cytochalasin inhibits the polymerization of actins by binding to the barbed ends of microfilaments and to actin dimers and monomers, with dissociation constants ranging from 2.6 to 19.1 μM (Goddette and Frieden, 1985; 1986). If cytochalasin binding to actin prevents ABP such as profilin from binding to actin monomers or to the barbed ends of actin filaments, cytochalasin could cause ABP to be released from actin species and therefore to become available for binding to PIP_2 and inhibiting polyphosphoinositide turnover, even in the presence of agonists that normally stimulate $[Ca^{2+}]_i$ mobilization. In turn, impaired $[Ca^{2+}]_i$ mobilization could prevent the remodeling of the actin network that is necessary for modulation of interactions between microfilaments and cell surface CD2. Phosphorylation of the ABP L-plastin has recently been shown to be regulated by stimulation of Jurkat T cells through CD2 (Henning et al., 1994), suggesting a second mechanism by which cell activation could be linked to remodeling of the actin network. These models, although still speculative, present attractive mechanisms by which intracellular signals, the structure of the actin network, and CD2 lateral mobility could be mutually regulated.

We do not observe an effect of colchicine on CD2 lateral mobility in either resting or activated cells. These results are consistent with reports that colchicine does not affect SER (Freed et al., 1989; Ishijima et al., 1991). We have previously shown that CD2 physically interacts with α/β tubulin, and that CD2-tubulin interactions are weakened by T cell activation induced by pairs of anti-CD2 mAbs (Offringa and Bierer, 1993). The observation that colchicine treatment does not affect the lateral mobility of CD2 in resting cells suggests that CD2-tubulin interactions at the plasma membrane may not be important in regulating CD2 mobility. It is not surprising that colchicine does not reverse the lateral immobilization of CD2 in activated cells, because T cell activation has been shown previously to decrease CD2-tubulin associations.

Intracellular cAMP increases upon binding of CD2 to its natural ligand CD58 or to the mAb TS2/18 (Hahn et al., 1991; Carrera et al., 1988). Further, this mediator regulates the avidity of CD2:CD58 (Hahn et al., 1993) and CD54:CD11a/CD18 (Dustin and Springer, 1989) interactions, as well as the increase in T cell adhesion stimulated by TCR/CD3 cross-linking (Dustin and Springer, 1989). Here we find that Db cAMP inhibits the cytoplasmic Ca^{2+} mobilization stimulated by pairs of anti-CD2 mAbs. Together with the observations that cAMP reduces diacylglycerol and inositol phosphate generation stimulated by pairs of anti-CD2 mAbs (Bismuth et al., 1988), these data suggest that cAMP inhibits inositol trisphosphate-mediated intracellular Ca^{2+} release. We also find that Db cAMP treatment partially prevents CD2 immobilization by pairs of anti-CD2 mAbs. The latter effect may be secondary to cAMP-mediated inhibition of Ca^{2+} mobilization. Elevation of intracellular cAMP causes actin depolymerization (Lamb et al., 1988; Hays and Lindberg, 1991; Egan et al., 1991), which appears to be mediated by cAMP-dependent protein kinase (Lamb et al., 1988). By inhibiting actin polymerization and microfilament network remodeling, increasing cAMP could modulate the immobilization of CD2 induced by cell activation.

In T cell hybridomas, CD2 ligation by CD58, single anti-CD2 mAbs, or pairs of anti-CD2 mAbs induces a transient (10–15 min) increase in intracellular [cAMP] (Hahn et al., 1991). Several considerations could explain the complete (90%) lateral immobilization of CD2 by pairs of anti-CD2 mAbs under conditions that might be expected to increase intracellular [cAMP]. First, the transient nature of the [cAMP] response to pairs of anti-CD2 mAbs may be responsible for the delay observed here (Fig. 2) between mAb treatment and both increased $[Ca^{2+}]_i$ and decreased CD2 lateral mobility. Second, the concentration of cAMP required to prevent CD2 immobilization by pairs of anti-CD2 mAbs (Table 2) may be greater than that induced by mAb treatment (Hahn et al., 1991, 1993).

Surface receptor lateral mobility may play a role in signal transduction. The fractional mobility of the vasopressin V_2-receptor increases linearly with vasopressin-induced cAMP production in LLC-PK_1 renal epithelial cells, suggesting a role for V_2-receptor mobility in vasopressin-mediated adenylate cyclase activation (Jans et al., 1991). Nerve growth factor receptors are preclustered and laterally immobile on responsive cells but diffusely distributed and laterally mobile on nonresponsive cells, suggesting that receptor immobilization is required for signal transduction (Venkatakrishnan et al., 1991). Here we demonstrate that CD2 lateral mobility is regulated by intracellular signals in Jurkat cells. Because changes in receptor mobility markedly affect CD2-mediated Jurkat cell adhesion strength (P.-Y. Chan et al.,

1991), regulation of surface receptor lateral movement by intracellular signals may serve as an important mechanism by which cell activation is coupled to increased adhesion strength.

Redistribution of adhesion molecules to contact areas increases the strength of adhesion between Jurkat cells and glass-supported planar membranes reconstituted with laterally mobile CD58 (P.-Y. Chan et al., 1991; Ferguson et al., 1991). Here we find that T cell activation through CD2 induces rapid mobilization of cytoplasmic Ca^{2+}, followed by lateral immobilization of cell surface CD2 molecules. During the 5–10 min interval between cytoplasmic Ca^{2+} mobilization and CD2 immobilization, anisotropic redistribution of CD2 molecules could occur. By increasing intracellular cAMP, CD58 ligation of cell surface CD2 at a contact site could prevent complete immobilization of CD2 and thereby allow increased numbers of CD2 molecules to diffuse to the contact area. Subsequent immobilization of CD2 could serve to maintain this adhesion molecule at high density at a contact area, and thereby to increase cellular adhesion strength.

The importance of intracellular signaling in regulating adhesion strength mediated by the CD2:CD58 receptor: ligand pair has been demonstrated by SER assays. SER, like treatment with pairs of anti-CD2 mAbs, causes an increase in $[Ca^{2+}]_i$ in T cells (Ledbetter et al., 1988). SER-associated intracellular Ca^{2+} mobilization could then induce CD2 immobilization. Inhibition of SER by pretreatment of T cells with A23187 in the presence of Ca^{2+} (Ishijima et al., 1991) could be due to CD2 immobilization induced by increased $[Ca^{2+}]_i$, which would prevent CD2 from diffusing to contact areas. In contrast, inhibition of SER by pretreatment of T cells with cytochalasin (Freed et al., 1989; Ishijima et al., 1991) could be due to inhibition of CD2 immobilization at contact sites by microfilament disruption. The timing of CD2 immobilization in relationship to T cell activation may determine whether adhesion is enhanced or inhibited.

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REFERENCES

Arulanandam, A. R., P. Moingeon, M. F. Concino, M. A. Recny, K. Kato, H. Yagita, S. Koyasu, and E. L. Reinherz. 1993. A soluble multimeric recombinant CD2 protein identifies CD48 as a low affinity ligand for human CD2: divergence of CD2 ligands during the evolution of humans and mice. *J. Exp. Med.* 177:1439–1450.

Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055–1069.

Bernard, A., R. W. Knowles, K. Naito, B. Dupont, B. Raynal, H. C. Tran, and L. Boumsell. 1986. A unique epitope on the CD2 molecule defined by the monoclonal antibody 9-1: epitope-specific modulation of the E-rosette receptor and effects on T-cell functions. *Hum. Immunol.* 17: 388–405.

Bierer, B. E., A. Peterson, J. Barbosa, B. Seed, and S. Burakoff. 1988a. Expression of the T-cell surface molecule CD2 and epitope-loss CD2 mutant to define the role of lymphocyte function-associated antigen 3 (LFA-3) in T-cell activation. *Proc. Natl. Acad. Sci. USA.* 85:1194–1198.

Bierer, B. E., A. Peterson, J. C. Gorga, S. H. Herrmann, and S. J. Burakoff. 1988b. Synergistic T cell activation via the physiological ligands for CD2 and the T cell receptor. *J. Exp. Med.* 168:1145–1156.

Bismuth, G., I. Theodorou, H. Gouy, S. Le Guovello, A. Bernard, and P. Debre. 1988. Cyclic AMP-mediated alteration of CD2 activation process in human T lymphocytes: preferential inhibition of the phosphoinositide cycle-related transduction pathway. *Eur. J. Immunol.* 18:1351–1357.

Brottier, P., L. Boumsell, C. Gelin, and A. Bernard. 1985. T cell activation via CD2 [T, gp50] molecules: accessory cells are required to trigger T cell activation via CD2-D66 plus CD2–9.6/$T11_1$ epitopes. *J. Immunol.* 135: 1624–1631.

Carpen, O., P. Pallai, D. E. Staunton, and T. A. Springer. 1992. Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and alpha-actinin. *J. Cell Biol.* 118:1223–1234.

Carrera, A. C., M. Rincon, M. O. De Landazuri, and M. Lopez-Botet. 1988. CD2 is involved in regulating cyclic AMP levels in T cells. *Eur. J. Immunol.* 18:961–964.

Chan, B. M., J. G. Wong, A. Rao, and M. E. Hemler. 1991. T cell receptor-dependent, antigen-specific stimulation of a murine T cell clone induces a transient, VLA protein-mediated binding to extracellular matrix. *J. Immunol.* 147:398–404.

Chan, P.-Y., M. B. Lawrence, M. L. Dustin, L. M. Ferguson, D. E. Golan, and T. A. Springer. 1991. Influence of receptor lateral mobility on adhesion strengthening between membranes containing LFA-3 and CD2. *J. Cell Biol.* 115:245–255.

Chopra, H., J. S. Hatfield, Y. S. Chang, I. M. Grossi, L. A. Fitzgerald, C. Y. O'Gara, L. J. Marnett, C. A. Diglio, J. D. Taylor, and K. V. Honn. 1988. Role of tumor cell cytoskeleton and membrane glycoprotein IRGpIIb/IIIa in platelet adhesion to tumor cell membrane and tumor cell-induced platelet aggregation. *Cancer Res.* 48:3787–3800.

Deckert, M., J. Kubar, D. Zoccola, G. Bernard-Pomier, P. Angelisova, V. Horejsi, and A. Bernard. 1992. CD59 molecule: a second ligand for CD2 in T cell adhesion. *Eur. J. Immunol.* 22:2943–2947.

Dustin, M. L., and T. A. Springer. 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature.* 341:619–624.

Egan, J. J., G. Gronowicz, and G. A. Rodan. 1991. Parathyroid hormone promotes the disassembly of cytoskeletal actin and myosin in cultured osteoblastic cells: mediation by cyclic AMP. *J. Cell. Biochem.* 45: 101–111.

Ferguson, L. M., M. L. Dustin, P.-Y. Chan, T. A. Springer, and D. E. Golan. 1991. Redistribution of GPI-linked LFA-3 to regions of contact between LFA-3 reconstituted planar bilayer membranes and $CD2^+$ T lymphoblasts. *J. Cell Biol.* 115:71*a*. (Abstr.)

Forscher, P. 1989. Calcium and polyphosphoinositide control of cytoskeletal dynamics. *Trends Neurosci.* 12:468–474.

Freed, B. M., N. Lempert, and D. A. Lawrence. 1989. The inhibitory effects of N-ethylmaleimide, colchicine and cytochalasins on human T-cell functions. *Int. J. Immunopharmacol.* 11:459–465.

Goddette, D. W., and C. Frieden. 1985. The binding of cytochalasin D to monomeric actin. *Biochem. Biophys. Res. Commun.* 128:1087–1092.

Goddette, D. W., and C. Frieden. 1986. The kinetics of cytochalasin D binding to monomeric actin. *J. Biol. Chem.* 261:15970–15973.

Golan, D. E., C. S. Brown, C. M. L. Cianci, S. T. Furlong, and J. P. Caulfield. 1986. Schistosomula of *Schistosoma mansoni* use lysophosphatidylcholine to lyse adherent human red blood cells and immobilize red cell membrane components. *J. Cell Biol.* 103:819–828.

Golding, J. W. 1976. Conjugation of antibodies with fluorochromes: modifications to the standard methods. *J. Immunol. Methods.* 13:215–226.

Goldschmidt-Clermont, P. J., J. W. Kim, L. M. Machesky, S. G. Rhee, and T. D. Pollard. 1991. Regulation of phospholipase C-ς1 by profilin and tyrosine phosphorylation. *Science.* 251:1231–1233.

Goldschmidt-Clermont, P. J., L. M. Machesky, J. J. Baldassare, and T. D. Pollard. 1990. The actin-binding protein profilin binds to PIP_2 and inhibits its hydrolysis by phospholipase C. *Science*. 247:1575–1578.

Hahn, W. C., S. J. Burakoff, and B. E. Bierer. 1993. Signal transduction pathways involved in T cell receptor-induced regulation of CD2 avidity for CD58. *J. Immunol.* 150:2607–2619.

Hahn, W. C., E. Menu, A. L. M. Bothwell, P. J. Sims, and B. E. Bierer. 1992a. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. *Science*. 256:1805–1807.

Hahn, W. C., Y. Rosenstein, S. J. Burakoff, and B. E. Bierer. 1991. Interaction of CD2 with its ligand lymphocyte function-associated antigen-3 induces adenosine 3′,5′-cyclic monophosphate production in T lymphocytes. *J. Immunol.* 147:14–21.

Hahn, W. C., Y. Rosenstein, V. Calvo, S. J. Burakoff, and B. E. Bierer. 1992b. A distinct cytoplasmic domain of CD2 regulates ligand avidity and T-cell responsiveness to antigen. *Proc. Natl. Acad. Sci. USA*. 89: 7179–7183.

Harlow, D. L. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 626 pp.

Hartwig, J. H., K. A. Chambers, and T. P. Stossel. 1989. Association of gelsolin with actin filaments and cell membranes of macrophages and platelets. *J. Cell Biol.* 108:467–479.

Hashemi, B., T. J. Feder, W. W. Webb, D. Holowka, and B. A. Baird. 1992. Diffusion and distribution measurements of the T cell receptor complex under stimulating and non-stimulating conditions. *Biophys. J.* 61:147*a*. (Abstr.)

Hays, R. M., and U. Lindberg. 1991. Actin depolymerization in the cyclic AMP-stimulated toad bladder epithelial cell, determined by the DNAse method. *FEBS Lett.* 280:397–399.

Henning, S. W., S. C. Meuer, and Y. Samstag. 1994. Serine phosphorylation of a 67-kDa protein in human T lymphocytes represents an accessory receptor-mediated signaling event. *J. Immunol.* 152:4804–4815.

Howard, F. D., J. A. Ledbetter, J. Wong, C. P. Bieber, E. B. Stinson, and L. A. Herzenberg. 1981. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. *J. Immunol.* 126:2117–2122.

Ishijima, S. A., H. Asakura, and T. Suzuta. 1991. Participation of cytoplasmic organelles in E-rosette formation. *Immunol. Cell Biol.* 69: 403–409.

Jaffe, S. H., D. R. Friedlander, F. Matsuzaki, K. L. Grossin, B. A. Cunningham, and G. M. Edelman. 1990. Differential effects of the cytoplasmic domains of cell adhesion molecules on cell aggregation and sorting-out. *Proc. Natl. Acad. Sci. USA*. 87:3589–3593.

Janmey, P. A., and T. P. Stossel. 1989. Gelsolin-polyphosphoinositide interaction: full expression of gelsolin-inhibiting function by polyphosphoinositides in vesicular form and inactivation by dilution, aggregation, or masking of the inositol head group. *J. Biol. Chem.* 264:4825–4831.

Jans, D. A., R. Peters, P. Jans, and F. Fahrenholz. 1991. Vasopressin V_2-receptor mobile fraction and ligand-dependent adenylate cyclase activity are directly correlated in LLC-PK_1 renal epithelial cells. *J. Cell Biol.* 114:53–60.

Johansson, B., M. P. Wymann, K. Holmgren, and K. E. Magnusson. 1993. *N*-formyl peptide receptors in human neutrophils display distinct membrane distribution and lateral mobility when labeled with agonist and antagonist. *J. Cell Biol.* 121:1281–1289.

Kabsch, W., and J. Vandekerckhove. 1992. Structure and function of actin. *Annu. Rev. Biophys. Biomol. Struct.* 21:49–76.

Kamoun, M., P. J. Martin, J. A. Hansen, M. A. Brown, A. W. Siadak, and R. C. Nowinski. 1981. Identification of a human T lymphocyte surface protein associated with the E-rosette receptor. *J. Exp. Med.* 153:207–212.

Kao, J. P. Y., A. T. Harootunian, and R. Y. Tsien. 1989. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J. Biol. Chem.* 264:8179–8184.

Kato, K., M. Koyanagi, H. Okada, T. Takanashi, Y. W. Wong, A. F. Williams, K. Okumura, and H. Yagita. 1992. CD48 is a counter-receptor for mouse CD2 and is involved in T cell activation. *J. Exp. Med.* 176: 1241–1249.

Koyasu, S., T. Lawton, D. Novick, M. A. Recny, R. F. Siliciano, B. P. Wallner, and E. L. Reinherz. 1990. Role of interaction of CD2 molecules with lymphocyte function-associated antigen 3 in T-cell recognition of nominal antigen. *Proc. Natl. Acad. Sci. USA*. 87:2603–2607.

Lamb, N. J. C., A. Fernandez, M. A. Conti, R. Adelstein, D. B. Glass, W. J. Welch, and J. R. Feramisco. 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. *J. Cell Biol.* 106: 1955–1971.

Lassing, I., and U. Lindberg. 1985. Specific interaction between phosphatidylinositol-4,5-bisphosphate and profilactin. *Nature*. 314: 472–474.

Ledbetter, J. A., P. S. Rabinovitch, I. Hellstrom, K. E. Hellstrom, L. S. Grosmaire, and C. H. June. 1988. Role of CD2 cross-linking in cytoplasmic calcium responses and T cell activation. *Eur. J. Immunol.* 18: 1601–1608.

Liu, S. J., and D. E. Golan. 1994a. Mobilization of intracellular free Ca^{2+} upon T cell activation via TCR/CD3 is required to induce a decrease in CD2 lateral mobility in Jurkat T cell membranes. *Biophys. J.* 66:149*a*. (Abstr.)

Liu, S. J., and D. E. Golan. 1994b. Regulation of CD2 lateral mobility by calmodulin and calcineurin in Jurkat T cell membranes. *Biophys. J.* 66: 149*a*. (Abstr.)

Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50kd T11 sheep erythrocyte receptor protein. *Cell.* 36:897–906.

Mookerjee, B. K., J. Cuppoletti, A. L. Rampal, and C. Y. Jung. 1981. The effects of cytochalasins on lymphocytes: identification of distinct cytochalasin-binding sites in relation to mitogenic response and hexose transport. *J. Biol. Chem.* 256:1290–1300.

Namba, Y., M. Ito, Y. Zu, K. Shigesada, and K. Maruyama. 1992. Human T cell L-plastin bundles actin filaments in a calcium-dependent manner. *J. Biochem. (Tokyo)*. 112:503–507.

Offringa, R., and B. E. Bierer. 1993. Association of CD2 with tubulin: evidence for a role of the cytoskeleton in T cell activation. *J. Biol. Chem.* 268:4979–4988.

O'Rourke, A. M., J. Rogers, and M. F. Mescher. 1990. Activated CD8 binding to class I protein mediated by the T-cell receptor results in signalling. *Nature*. 346:187–189.

Pacaud, M., and J. Derancourt. 1993. Purification and further characterization of macrophage 70-kDa protein, a calcium-regulated, actin-binding protein identical to L-plastin. *Biochemistry*. 32:3448–3455.

Papadogiannakis, N., T. E. Nordstrom, L. C. Andersson, and C. H. J. Wolff. 1989. cAMP inhibits the OKT3-induced increase in cytoplasmic free calcium in the Jurkat T cell line: the degree of inhibition correlates inversely with the amount of CD3 binding ligand used. *Eur. J. Immunol.* 19:1953–1956.

Peterson, A., and B. Seed. 1987. Monoclonal antibody and ligand binding sites of the T cell erythrocyte receptor (CD2). *Nature*. 329: 842–846.

Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins: a critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55:987–1035.

Sanchez-Madrid, F., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff, and T. A. Springer. 1982. Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. *Proc. Natl. Acad. Sci. USA*. 79: 7489–7493.

Shimizu, Y., G. A. Van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (beta 1) integrin receptors on T cells. *Nature*. 345:250–253.

Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature*. 346:425–434.

Stolpen, A. H., D. E. Golan, and J. S. Pober. 1988. Tumor necrosis factor and immune interferon act in concert to slow the lateral diffusion of proteins and lipids in human endothelial cell membranes. *J. Cell Biol.* 107:781–789.

Thatte, H. S., K. R. Bridges, and D. E. Golan. 1994. Microtubule inhibitors differentially affect translational movement, cell surface expression, and endocytosis of transferrin receptors in K562 cells. *J. Cell. Physiol.* 160: 345–357.

Van Kooyk, Y., P. van de Wiel-van Kemenade, P. Weder, T. W. Kuijpers, and C. G. Figdor. 1989. Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature*. 342: 811–813.

Van Wauwe, J., J. Goossens, W. Decock, P. Kung, and G. Goldstein. 1981. Suppression of human T-cell mitogenesis and E-rosette formation by the monoclonal antibody OKT11A. *Immunology*. 44:865–871.

Venkatakrishnan, G., C. A. McKinnon, C. G. Pilapil, D. E. Wolf, and A. H. Ross. 1991. Nerve growth factor receptors are preaggregated and immobile on responsive cells. *Biochemistry*. 30:2748–2753.

Wallace, D. L., E. A. MacIntyre, D. C. Linch, and P. C. L. Beverley. 1987. Investigation of the signals induced by stimulation of the CD2 pathway of T-cell activation. *In* Leucocyte Typing III. White Cell Differentiation Antigens. A. J. McMichael, P. C. L. Beverley, S. Cobbold, M. J. Crumpton, W. Gilks, F. M. Gotch, N. Hogg, M. Horton, N. Ling, I. C. M. MacLennan, D. Y. Mason, C. Milstein, D. Spiegelhalter, and H. Waldmann, editors. Oxford University Press, Oxford. 120–122.

Yang, S. Y., S. Chouaib, and B. Dupont. 1986. A common pathway for T lymphocyte activation involving both the CD3-Ti complex and CD2 sheep erythrocyte receptor determinants. *J. Immunol*. 137: 1097–1100.

Yang, S. Y., S. Rhee, G. Angelos, and B. Dupont. 1987. Functional analysis of CD2 (T, p50) epitopes detected by 24 CD2 antibodies. *In* Leucocyte Typing III. White cell Differentiation Antigens. A. J. McMichael, P. C. L. Beverley, S. Cobbold, M. J. Crumpton, W. Gilks, F. M. Gotch, N. Hogg, M. Horton, N. Ling, I. C. M. MacLennan, D. Y. Mason, C. Milstein, D. Spiegelhalter, and H. Waldmann, editors. Oxford University Press, Oxford. 113–116.