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Differential Activation of Phosphotyrosine Protein Phosphatase Activity in a Murine T Cell Hybridoma by Monoclonal Antibodies to CD45*

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Samuel J. Goldman‡§, Shashi Uniyal‡, Laura M. Ferguson§¶‡‡, David E. Golan¶, Steven J. Burakoff‡, and Peter A. Kiener‖**

From the ‡Division of Pediatric Oncology, Dana Farber Cancer Institute, the ¶Departments of Medicine and of Biological Chemistry and Molecular Pharmacolody, Harvard Medical School, Boston, Massachusetts 02115 and ‖Bristol-Myers Squibb, Pharmaceutical Research Institute, Wallingford, Connecticut 06492

The effect of two anti-CD45 (T200, LCA, Ly5) antibodies on the activation of the murine T-cell hybridoma 13.13 has been evaluated. These studies have been carried out in a system that did not require cross-linking or coclustering of antibodies. Activation of 13.13 cells with the anti-CD3 monoclonal antibody, 145.2C11, gave rise to rapid increases in intracellular calcium and interleukin-2 production. Additionally, within 1 min, phosphorylation on tyrosine of four major proteins of about 130,000, 110,000, 80,000, and 37,000 daltons could be seen. Pretreatment of the cells with the anti-CD45 mAb M1/89.18.7.HK markedly inhibited all three biological responses, while an alternate anti-CD45 antibody, M1/9.3.4.HL.2, had little effect. The two antibodies bound to CD45 with similar affinities, and no differences in the lateral mobility of antibody-CD45 complexes in the cell membrane were observed. The inhibition of activation of the cells by M1/89.18.7.HK was abrogated significantly both by the phosphotyrosine protein phosphatase inhibitor orthovanadate and by excess M1/9.3.4.HL.2. If M1/89.18.7.HK was added to the 13.13 cells after they had already been activated with anti-CD3, it very effectively stimulated dephosphorylation of substrates that had been phosphorylated on tyrosines prior to adding the anti-CD45 antibody. These results indicate that the phosphotyrosine protein phosphatase activity of CD45 is critical to its biological function and that bivalent (*i.e.* uncross-linked) anti-CD45 antibodies can give rise to markedly different responses. One of the antibodies, M1/89.18.7.HK, appears to behave much like a receptor ligand and is able to activate the enzymatic activity associated with the CD45 transmembrane protein.

The CD45 antigens, also referred to as leukocyte common antigens, T200, and Ly5 comprise a heterogeneous family of transmembrane glycoproteins that is present on most cells of the hematopoietic lineage. The members of this family range in molecular mass from 180 to 220 kDa and vary in both protein sequence and glycosylation. Differences in amino acid sequence of CD45 isoforms result from alternative splicing of exons that encode the 400–500 amino acids of the extracellular domain of the protein (reviewed in Ref. 1). In contrast, the large intracellular domain of 705 amino acids is highly conserved. Two domains of the cytoplasmic region of CD45 have significant sequence homology to a human placental phosphatase (2), and recently CD45 has been shown to possess intrinsic protein tyrosine phosphatase activity (3, 4).

Studies have shown that mAbs[1] to CD45 can either block or augment various lymphocyte functions (4–9). It has also been shown that it is possible to overcome the inhibition of activation by mAb to CD45 by pretreating the cells with the PTPase inhibitor sodium orthovanadate (4). Recently, it has been observed that mutant T lymphocytes that do not possess CD45 could not be stimulated to proliferate in response to antigen, and were unable to mobilize cellular Ca^{2+} or activate phosphotidylinositol turnover (10, 11). Together, these results suggest that the CD45 phosphatase activity is an integral component of cellular signal transduction pathways in lymphocytes.

Although it is clear that CD45 can have profound influence on T-cell function, the manner in which CD45 tyrosine phosphatase activity exerts these effects is not completely understood. Interactions between CD45 and several T-cell surface molecules have been demonstrated by studies using mAbs against CD45 cross-linked to anti-CD3 or anti-CD2 mAbs (8, 12–15). Consistent with these observations, recent evidence has indicated that mAbs against CD45 can, under certain circumstances, inhibit anti-CD2-stimulated phosphorylation, phosphotidylinositol turnover, and Ca^{2+} mobilization (15). In contrast, it has also been shown that some anti-CD45 mAbs can enhance anti-CD2-stimulated T-cell proliferation (13). A possible molecular basis for CD45 enhancement and/or inhibition of CD2 stimulation was suggested by the recent observation that CD45 could physically associate with CD2 on the surface of human lymphocytes (16). Association between CD45 and CD4 has also been implicated by *in vitro* and *in*

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** To whom correspondence should be addressed: Dept. of Immunology 205, Bristol-Myers Squibb, 5 Research Pkwy., Wallingford, CT 06492. Tel.: 203-284-6404; Fax: 203-284-7569.

§ Dept. of Preclinical Biology Genetics Institute, Cambridge, MA 02410.

‡‡ Connaught Laboratories, Swiftwater, PA 18370.

[1] The abbreviations used are: mAb, monoclonal antibody; PTPase, phosphotyrosine protein phosphatase; IL-2, interleukin-2; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin; FPR, fluorescence photobleaching recovery; TcR, T-cell receptor.

situ experiments that show the CD4-associated tyrosine kinase $p56^{lck}$ can serve as a substrate for the CD45 tyrosine phosphatase activity (17, 18) and that coclustering of CD45 with CD4 alters the phosphorylation and kinase activity of $p56^{lck}$ (17). More recently it has been shown by fluorescence resonance energy transfer and immunoprecipitation studies on T-cells that 48–72 h after activation both CD4 and CD8 can become associated with CD45.[2]

In studies to date, it has not been clear how CD45 PTPase interacts with the intracellular signalling network, and interpretation of results is complicated because many systems require cross-linking of cell surface antigens to demonstrate CD45 activity. Furthermore, although CD45 has a "receptor-like" structure, the ligand has not been identified, and it has not been shown that PTPase activity can be stimulated upon binding of mAbs.

In this report we have examined the effects of two anti-CD45 mAbs, designated M1/9.3 and M1/89, on T-cell activation in a murine T-cell hybridoma 13.13. The mAbs bound with similar affinities, and no differences could be detected in mobility in the cell membrane of the mAb/CD45 complexes. The M1/89 mAb very strongly inhibited anti-CD3-mediated Ca^{2+} mobilization, IL-2 production, and protein tyrosine phosphorylation, while M1/9.3 had little effect on any of these parameters. Additionally M1/89 decreased tyrosine phosphorylation of intracellular proteins even when it was added several min after stimulation of the cells with anti-CD3. These effects were seen in the absence of any secondary cross-linking or co-cross-linking of mAbs. These data show that different mAbs against CD45 can be functionally distinct, and suggest that the inhibition of T-cell activation by the M1/89 anti-CD45 mAb occurs by the stimulation of PTPase activity.

MATERIALS AND METHODS

Antibodies and Cell Lines—Hybridomas producing antibodies to CD45 were obtained from the ATCC; the clones were M1/9.3.3.4.HL.2 (M1/9.3, rat IgG2a) and M1/89.18.7.HK (M1/89, rat IgG2b). The hybridoma 145.2C11 producing mAb to CD3 was kindly provided by Dr. J. Bluestone (University of Chicago). Antibodies were purified from ascites or culture supernatants by protein A or protein G affinity chromatography. The murine T-cell hybridoma, 13.13, has been described previously (19).

Fluorescent Labeling of Antibodies and Binding Assays—The purified antibodies were labeled with fluoroscein isothiocyanate (FITC) and purified by gel filtration on Sephadex G-25. The binding of the FITC-mAbs was carried out in PBS supplemented with 2% fetal bovine serum and 0.02% sodium azide at 4 °C for 30 min in the presence of various concentrations of unlabeled antibody. In experiments looking at the effect of anti-CD45 mAb on the binding of anti CD3 mAb, cells were incubated with M1/89 or FITC-2C11 at 4 °C for 30 min in PBS/fetal bovine serum, washed, and then incubated with the appropriate second mAb for an additional 30 min at 4 °C. The cells were then washed twice and analyzed. Bound FITC-labeled antibody was quantitated by measuring the mean fluorescence on a FACSCAN analyzer.

Radiolabeling of Cells, Immunoprecipitations, and SDS-PAGE—Cells were surface labeled with ^{125}iodine by standard procedures using lactoperoxidase (20). After labeling, the cells were washed four times in PBS and then lysed in lysis buffer (20 mM HEPES, 1.0% Triton X-100, 5 mM PO_4, 150 mM NaCl, 10 mM pyrophosphate, 5 mM orthovanadate, 25 mM NaF, 1 mM $ZnCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 20 μg/ml each of soybean trypsin inhibitor, antipain, leupeptin, and pepstatin A, pH 7.4) for 20 min at 0 °C. The supernatants of the lysed cells were clarified by centrifugation at 15,000 × *g* for 30 min and then precleared by incubation with IgGsorb (The Enzyme Center, Malden, MA) followed by a nonreactive preformed complex of mouse IgG_1 with goat anti-mouse IgG serum as described previously (21). Specific antigens were then precipitated from the cleared supernatants by incubation with preformed complexes of the appropriate antibody with goat anti-rat IgG serum for 2 h at 4 °C. The immunoprecipitates were then pelleted at 15,000 × *g* for 20 min. The supernatants were then extensively cleared (four changes over 2 days) with preformed complexes of the same mAb prior to a final precipitation with a preformed complex of the alternate anti-CD45 mAb, for 2 h at 4 °C. These immunoprecipitates were pelleted as before. All precipitates were washed four times in lysis buffer followed by pelleting through a discontinous sucrose gradient (400 μl of 10% sucrose, 700 μl of 20% sucrose, in lysis buffer). The precipitates were then washed a further two times prior to being solubilized in 200 μl of SDS sample buffer at 100 °C for 10 min. Samples were then analyzed by separation by SDS-PAGE on 4–20% gradient gels and autoradiography.

Calcium Mobilization—Intracellular Ca^{2+} was quantitated using the fluorescent probe indo-1 (Molecular Probes, Eugene, OR). Cells at 1 × 10^6/ml were loaded by incubation in buffer A (140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM glucose, 2 mM glutamine, 0.1% BSA, and 10 mM HEPES, pH 7.4) for 15–30 min with 1 μM of the acetoxymethylester of indo-1. After the cells were washed in buffer A, they were resuspended at 5 × 10^6/ml. Indo-1-loaded cells were kept at room temperature until use. For assay of intracellular Ca^{2+}, cells were diluted to 5–7 × 10^5/ml in buffer A and allowed to equilibate at 37 °C for 5 min. Changes in fluoresence intensity were measured using a SLM Aminco model SPF 500C spectrofluorimeter with excitation set at 340 nm. Emission was monitored at 405 and 480 nm sequentially, using the SLM software, with a data point plotted every 8 s. Excitation and emission slit widths were 5 and 7.5 nm, respectively. The sample was stirred continuously and temperature was maintained at 37 °C. Intracellular Ca^{2+} is expressed as the ratio of emission at 405/480 on an arbitrary scale.

Detection of Phosphotyrosine in Cell Extracts—Cells (1.25 × 10^7) in 500 μl of Hanks' balanced salt solution, containing 20 mM HEPES and 0.075% sodium bicarbonate, pH 7.4, were incubated with the various antibodies at 37 °C for the indicated times. All reactions were initiated by the addition of 2C11. The reactions were stopped by the quenching with 750 ml of ice-cold PBS containing 500 μM of orthovanadate, and the cells were immediately spun at 5000 rpm for 30 s. The supernatants were then removed and the cells incubated with 150 μl of lysis buffer (20 mM HEPES, 1.0% Triton X-100, 5 mM PO_4, 150 mM NaCl, 10 mM pyrophosphate, 5 mM orthovanadate, 25 mM NaF, 1 mM $ZnCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 20 μg/ml each of soybean trypsin inhibitor, antipain, leupeptin, and pepstatin A, pH 7.4) for 20 min on ice. The lysates were spun at 15,000 rpm for 15 min at 4 °C, and the supernatants added to equal volumes of 2 × SDS sample buffer. The proteins were then separated by SDS-PAGE on 10–20% gradient gels and then transferred by Western blotting to Hybond C Extra membranes (Amersham). The membranes were blocked with PBS containing 5% BSA, 1% ovalbumin, 0.05% sodium azide, and 500 μM vanadate for 16 h at 4 °C. Proteins containing phosphotyrosine were detected with a polyclonal anti-phosphotyrosine antibody (22) followed by ^{125}I-protein A (ICN). Phosphotyrosine-containing proteins were visualized by autoradiography using Kodak X-Omat AR x-ray film. Bands on the autoradiographs were quantitated by scanning on an LKB laser densitometer.

IL-2 Production—IL-2 production in the T-cell hybridomas following stimulation by antibodies was followed by measuring the ability of the supernatants to support the proliferation of the IL-2-dependent murine T-cell line CTLL-20. The 13.13 cells were treated with various antibodies at 37 °C for 20 h. The supernatants were harvested, frozen, and thawed once to lyse any remaining viable cells, titrated by serial 2-fold dilutions, and then assayed for their ability to support the proliferation of CTLL-20 cells. Proliferation of these cells was measured by assaying the formation of the formazan product of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma). The results are expressed relative to a recombinant IL-2 standard (Amgen) with half-maximal proliferation defined as 100 units of IL-2 per ml. The data shown are the average (± S.D.) of two samples, each assayed in duplicate.

Fluorescence Photobleaching Recovery—Fluorescence photobleaching recovery (FRP) is used to measure the lateral mobility of labeled proteins and lipids in membranes (23). Briefly, a Gaussian laser beam is focused to a waist at the sample plane of a fluorescent microsscope. After a brief, intense photobleaching pulse, recovery of fluorescence is monitored by periodic low intensity pulses. Recovery results from the lateral diffusion of unbleached fluorophores into the bleached area. Nonlinear least-squares analysis of fluorescence recovery data yields both the diffusion coefficient (D) and the fraction (f) of

[2] R. S. Mittler, B. M. Rankin, and P. A. Kiener, submitted for publication.

fluorescently labeled molecules that are free to diffuse on the time scale of the experiment.

Cells were washed in FPR buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.5% (w/v) BSA, pH 7.6). FITC-conjugated CD45 mAbs were centrifuged in a Beckman Airfuge at 27 p.s.i., for 30 min, at 4 °C. Cells were incubated with mAb for 20 min at 4 °C, washed and resuspended in the above buffer with the pH adjusted to 7.8. Samples were placed in a 37 °C water bath for 10 min. Where appropriate, 2C11 was added just prior to placing the cells on slides coated with buffer containing 1% BSA. All data were recorded at room temperature. Bleaching pulses were typically 30 ms in duration using a beam power of 150 μW. Measuring pulses were 4 μs in duration using a typical beam power of 20 μW. Measuring intensities and amplification settings were adjusted such that the signal from unlabeled cells was <5% of the signal from labeled cells. Fluorescence recovery was typically monitored for 100 s. The Gaussian beam radius was 1.04 ± 0.13 μm, as determined by scanning 0.22-μm diameter fluorescent beads (Polysciences Inc., Warrington, PA) immobilized in saturated Airvol U-205 (Air Products, Allentown, PA) in glycerol and calculating the average $1/e^2$ radius of the fluorescent profile of 21 beads. All data was analyzed on a Meridian Instruments ACAS 570 interactive laser cytometer.

RESULTS

Characterization of the M1/9.3 and M1/89 mAbs—Immunoprecipitation of ^{125}I surface labeled cells with the M1/9.3 and M1/89 mAbs was used to determine which CD45 isoform each mAb recognized (Fig. 1). Both the M1/9.3 and the M1/89 mAbs immunoprecipitated identical patterns of isoforms of CD45, a major doublet of molecular mass from 180 to 210 kDa and minor bands of 150 kDa (*lanes 2* and *6*). Thus, it appeared that neither of these mAbs identified restricted or unique epitopes that were present only on particular CD45 isoforms. The cell lysates were then extensively cleared with either M1/9.3 or M1/89 such that no radioactivity could be detected in the final immunoprecipitate (Fig. 1, *lanes 3* and *7*), and this was then followed by immunoprecipitation with the alternate CD45 mAb. No specific radioactivity was detected in these crossover immunoprecipitations (*lanes 4* and *8*) confirming that neither of the two antibodies recognized a species that was not also recognized by the other. The two antibodies also immunoprecipitated PTPase activity that could hydrolyze both *p*-nitrophenyl phosphate and [^{32}P]phosphotyrosine reduced carboxymethylated and maleylated lysozyme (data not shown).

To measure the relative affinities of the two mAbs for CD45 and the ability of each mAb to compete with the other for binding, 13.13 cells were incubated with a fixed concentration of either FITC-labeled M1/9.3 or FITC-M1/89 in the presence of various concentrations of unlabeled mAbs. The ability of the unlabeled antibodies to compete with the FITC-mAb was followed by measuring the mean fluorescence of the cells in a FACSCAN analyzer (Fig. 2, *A* and *B*). Unlabeled M1/9.3 and M1/89 were equally effective in competing for binding of FITC-M1/9.3 (Fig. 2*A*). Similar results were seen when unlabeled M1/9.3 and M1/89 were used to compete for binding of FITC-M1/89 (Fig. 2*B*). These data suggest that the M1/89 and M1/9.3 mAbs identify epitopes that are in close proximity to each other and confirm that they recognize the same distribution of CD45 isoforms. Furthermore, the observation that the competition curves for cross-blocking were essentially equivalent for both M1/9.3 and M1/89 suggests that the affinity of the mAbs for CD45 is very similar.

Effect of Anti-CD45 mAbs on CD45 Lateral Mobility—The binding of a mAb to a cell surface protein may affect the freedom of movement of the protein in the cell membrane. Loss of mobility could account for inhibition of an essential protein activity or could influence how the antigen interacts with a target protein. To address this possibility, we used fluorescence recovery after photobleaching (FPR) to determine the effects of the two mAbs on the lateral mobility of

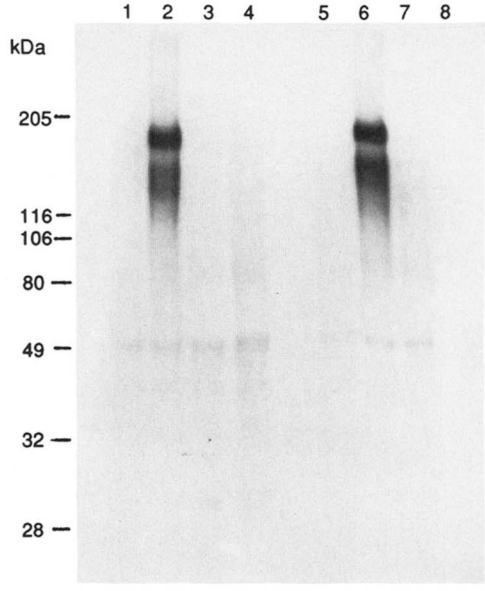

FIG. 1. **SDS-PAGE analysis of immunoprecipitates of anti-CD45 mAbs from ^{125}I-labeled 13.13 cells.** Immunoprecipitations were carried out as described in the text. *Lanes 1* and *5*, precleared with nonrelevent preformed complex; *lane 2*, initial M1/9.3 immunoprecipitate; *lane 3*, M1/9.3 final immunoprecipitate following extensive clearing; *lane 4*, supernatant from *lane 3* sample immunoprecipitated with M1/89; *lane 6*, initial M1/89 immunoprecipitate; *lane 7*, M1/89 final immunopreciptate following extensive clearing; *lane 8*, supernatant from *lane 7* sample immunoprecipitated with M1/9.3.

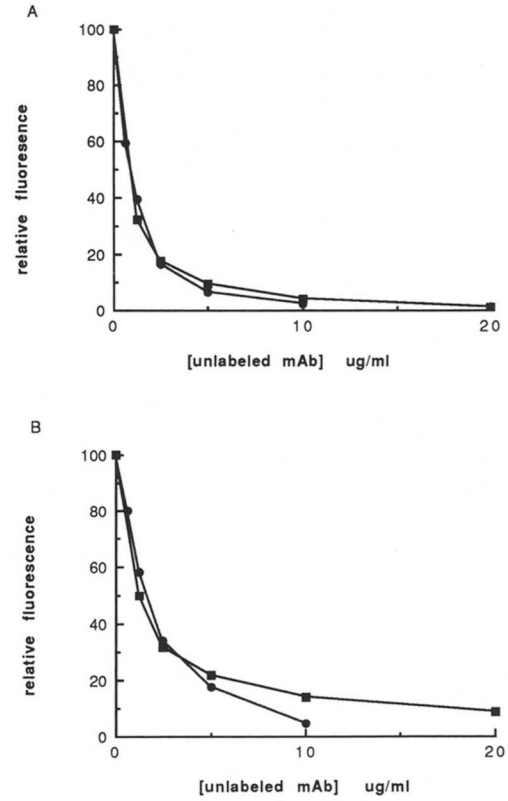

FIG. 2. **Analysis of binding of FITC-labeled anti-CD45 mAbs to 13.13 cells.** *A*, binding of FITC-M1/9.3, 620 ng/ml, in the presence of various concentrations of unlabeled M1/9.3 (■) and M1/89 (●). *B*, binding of FITC-M1/89, 310 ng/ml, in the presence of various concentrations of unlabeled M1/9.3 (■) and M1/89 (●).

CD45 in the cell membrane. This technique quantitates both the diffusion coefficient (D), a measure of lateral mobility, and the fraction (f) of the fluorescently labeled molecules that are free to diffuse in the time scale of the experiment. The diffusion coefficient for CD45 and the fractional recovery were not significantly different when measured using fluoresceinated M1/9.3 or fluoresceinated M1/89 (Table I). Furthermore, stimulation of the 13.13 hybridoma by 2C11 prior to measuring lateral mobility did not change the value of D or f compared with the values measured on unstimulated hybridomas. These data indicate that neither the M1/9.3 nor the M1/89 mAb immobilized the CD45 molecule in the cell membrane such that it would be unable to interact with substrates. In addition, on average, there was no measurable difference in the mobility of the CD45 molecule when bound by M1/9.3 or M1/89 or after cell activation with 2C11.

Effect of Anti-CD45 mAbs on Anti-CD3-stimulated IL-2 Production—The 13.13 T-cell hybridomas produce IL-2 in response to stimulation by soluble anti-CD3 mAb (19). We examined the effect of the two anti-CD45 mAbs on anti-CD3 (145-2C11)-stimulated 13.13 hybridomas (Fig. 3). M1/89 profoundly inhibited anti-CD3-stimulated IL-2 production. The inhibition by M1/89 could be detected at concentrations as low as 10–20 ng/ml, and maximal inhibition was seen above 1 μg/ml. In five independent experiments, the range of maximal inhibition by M1/89 was 71–87%. In contrast, M1/9.3, at best, only slightly inhibited anti-CD3-stimulated IL-2 production. In seven independent experiments, the range of maximal inhibition by M1/9.3 was 2–22%. These results indicate that the M1/89 CD45 mAb was much more effective in inhibiting anti-CD3-stimulated IL-2 production than was M1/9.3. Identical results to those described above were seen in the BY155.16, the parent hybridoma to 13.13, indicating that expression of the human CD4 gene product in the 13.13 hybridoma did not alter CD45 function (data not shown).

Effect of Anti-CD45 mAbs on Anti-CD3-stimulated Mobilization of Cellular Ca^{2+}—Anti-CD3 mAb has been shown to stimulate cellular Ca^{2+} mobilization in the 13.13 hybridoma. We analyzed the effect of M1/9.3 and M1/89 on this early signal of T-cell activation. Addition of soluble 2C11 mAb stimulated an increase in cellular Ca^{2+} level in the 13.13 hybridoma (Fig. 4*A*). Preincubation of the 13.13 cells with M1/89 several minutes prior to addition of 2C11 almost totally blocked the increase in cellular Ca^{2+} (Fig. 4*B*). In contrast, preincubation of the 13.13 cells with M1/9.3 mAb had no effect on the ability of 2C11 to stimulate an increase in cellular Ca^{2+} (Fig. 4*C*).

Binding of Anti-CD3 mAb in the Presence of M1/89—To determine whether the inhibition of anti-CD3-induced Ca^{2+} mobilization and IL-2 production by M1/89 could be attributed to disruption of the binding of 2C11 to CD3, cells were treated with M1/89 and the binding of FITC-2C11 assessed by measuring the mean fluorescence on a FACSCAN analyzer. Treatment of the cells with 1 μg/ml M1/89 for 30 min at 4 °C, either before or after incubation with FITC-2C11 (1 μg/ml), did not alter the binding of the FITC-labeled antibody (Fig. 5, *A* and *B*) indicating that this anti-CD45 mAb did not abrogate the binding of 2C11 and also did not induce a rapid dissociation of antigen-bound mAb.

Effect of Anti-CD45 mAbs on Anti-CD3-stimulated Tyrosine

TABLE I

Lateral mobility of mAb/CD45 complexes in 13.13 cell membranes

13.13 cells were labelled with FITC-conjugated anti-CD45 mAb and lateral mobility measured using the FPR technique. In some cases cells were stimulated by treatment with 2C11 mAb prior to FPR measurements. D, diffusion coefficient, $\times 10^{10}$ cm^2 s^{-1}. f, fractional mobility, %. n, number of cells. Values are expressed as mean ± S.D.

mAbs	D	f	n
FITC-M1/89	3.8 ± 3.0	50 ± 17	33
FITC-M1/89 + 2C11	4.7 ± 3.1	53 ± 20	33
FITC-M1/9.3	4.8 ± 2.8	50 ± 18	39
FITC-M1/9.3 + 2C11	4.1 ± 2.4	48 ± 19	33

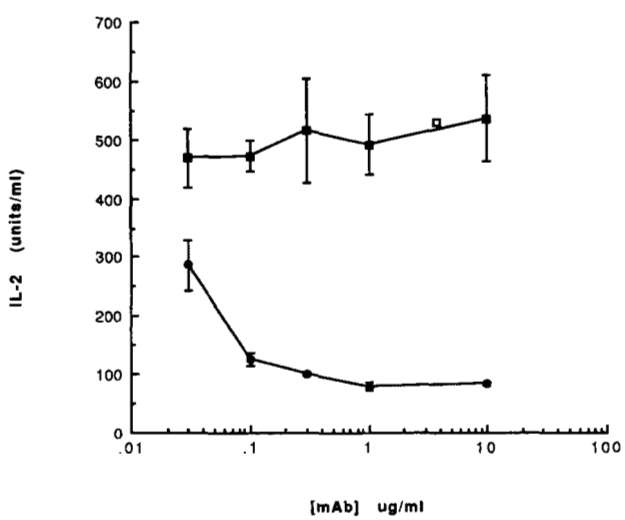

FIG. 3. **Influence of anti-CD45 mAbs on 2C11-stimulated IL-2 production by 13.13 cells.** Cells were incubated with the various mAbs as described under "Materials and Methods." ●, M1/89; ■, M1/9.3; □, rat IgG 5 μg/ml.

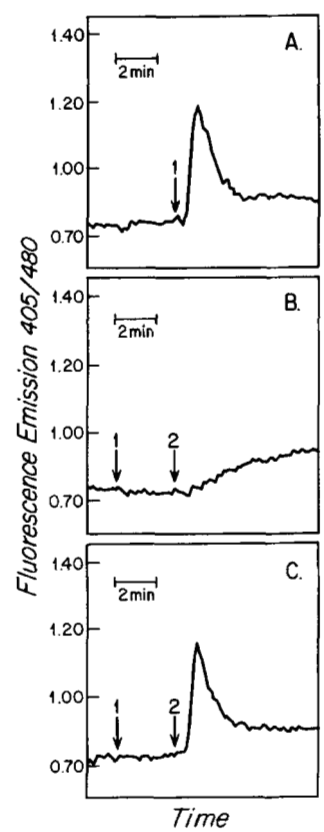

FIG. 4. **Influence of anti-CD45 mAbs on 2C11-stimulated Ca^{2+} mobilization.** *A*, 2C11 (500 ng/ml) added at 1. *B*, pretreatment with M1/89 (2 μg/ml, added at *1*) followed by 500 ng/ml 2C11, added at 2. *C*, pretreatment with M1/9.3 (2 μg/ml, added at *1*) followed by 500 ng/ml 2C11, added at 2.

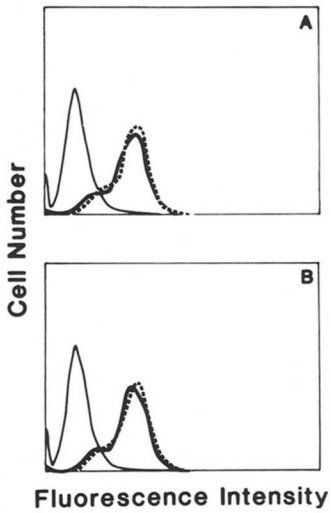

FIG. 5. **Analysis of binding of FITC-2C11 to the 13.13 hybridoma.** *A*, FITC hamster IgG isotype control (1 μg/ml) (*thin line*); FITC-2C11 (1 μg/ml) (*thick line*); pretreatment with 1 μg/ml M1/89 prior to addition of FITC-2C11. *B*, FITC hamster IgG isotype control (1 μg/ml) (*thin line*); FITC-2C11 (1 μg/ml) (*thick line*); treated with 1 μg/ml M1/89 for 30 min following binding of FITC-2C11 (*dotted line*). Incubations were carried out as described under "Materials and Methods."

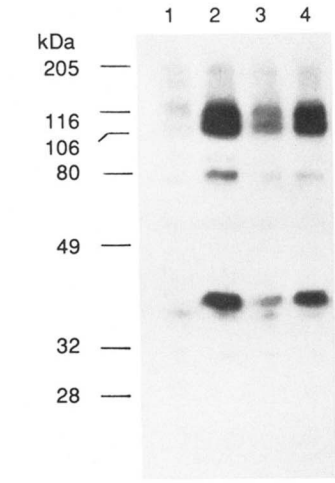

FIG. 6. **Tyrosine phosphorylation in the 13.13 hybridoma following treatment with various mAbs.** Samples were taken 5 min after activation with 2C11. *Lane 1*, no additions; *lane 2*, 500 ng/ml 2C11; *lane 3*, 2 μg/ml M1/89 for 1 min followed by 500 ng/ml 2C11; *lane 4*, 2 μg/ml M1/9.3 for 1 min followed by 500 ng/ml 2C11.

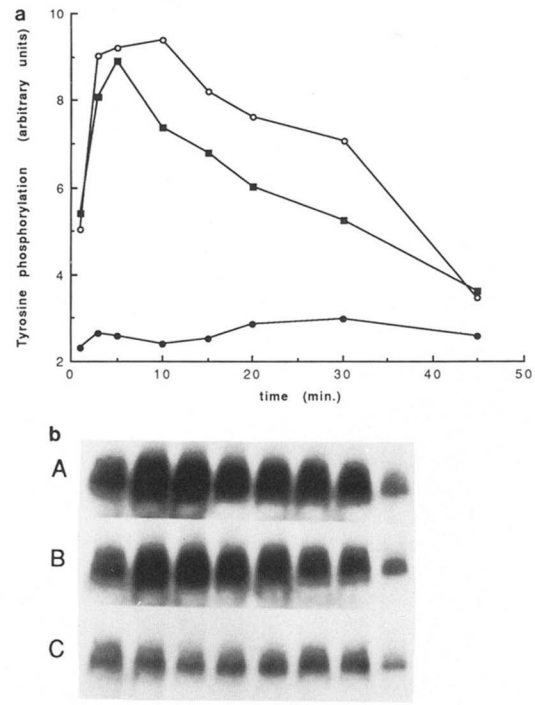

FIG. 7. **Kinetics of phosphorylation of 110–130-kDa doublet following stimulation with 2C11.** In *a*: ○, 2C11 (500 ng/ml); ●, M1/89 (2 μg/ml) for 1 min followed by 2C11 (500 ng/ml); ■, M1/9.3 (2 μg/ml) for 1 min followed by 2C11 (500 ng/ml). The data are from one experiment; similar results were obtained in three other experiments. In *b*, autoradiographs of 110–130-kDa doublet used in Fig. 6. *A*, 2C11; *B*, M1/9.3 + 2C11; *C*, M1/89 + 2C11.

Phosphorylation—Stimulation of T-cells through CD3 gives rise to a rapid increase in protein tyrosine phosphorylation. Since CD45 has recently been shown to possess intrinsic protein tyrosine phosphatase activity, we examined the effect of M1/9.3 and M1/89 on anti-CD3-stimulated protein tyrosine phosphorylation. Stimulation of the 13.13 T-cell hybridoma with 2C11 mAb (500 ng/ml) resulted in a rapid increase in the tyrosine phosphorylation of several protein bands (Fig. 6, *lane 2*, and Fig. 9, *lane 2*). The major bands included a doublet at about 110–130 kDa, a band at 80 kDa, and a band at 37 kDa. The specificity of the anti-phosphotyrosine antibody was confirmed by experiments showing that phosphotyrosine and phenylphosphate, but not phosphoserine or phosphothreonine inhibited antibody binding to the protein blots (data not shown). Phosphorylation was rapid, being evident within 1 min, and reaching a maximum of a 5–6-fold increase within 5–10 min. After 5–10 min the level of phosphorylation started to decrease so that 30–45 min after stimulation the levels were comparable with those of unstimulated cells. The time course for the phosphorylation and subsequent dephosphorylation of the 110–130 kDa doublet is shown in Fig. 7. The kinetics of the phosphorylation and dephosphorylation of the other bands was found to be similar (data not shown). Pretreatment of cells with 2 μg/ml of M1/89 for 1 min resulted in marked changes in 2C11-induced tyrosine phosphorylation. The mAb almost completely blocked the 2C11-induced increase in tyrosine phosphorylation of all the protein bands throughout the incubation (Figs. 6 and 7). In contrast, pretreatment of cells with 2 μg/ml M1/9.3, had little effect on 2C11-induced increases (4–5-fold) in tyrosine phosphorylation of the major bands (Fig. 6, *lane 4*, and Fig. 9, *lane 3*) and on the kinetics of phosphorylation of the proteins (Fig. 7).

Pretreatment of the T-cell hybridoma for 1 min with 2 μg/ml of M1/89, together with increasing concentration of M1/9.3, followed by activation with 500 ng/ml 2C11, gave rise to dose dependent restoration in the levels of tyrosine phosphorylation of the proteins (Fig. 8). These data are consistent with the earlier observation that M1/9.3 can compete with M1/89 for binding to CD45, and moreover, even at high concentrations of M1/9.3, signalling through CD3 still occurred. Similarly, preincubating the cell with the PTPase inhibitor sodium orthovanadate for 30 min at 37 °C before the addition of M1/89 and 2C11 partially restored, in a dose-dependent manner, the levels of tyrosine phosphorylation to those stimulated by 2C11 alone (Table II). At 100, 50, and 25 μM vanadate the levels of phosphorylation of the doublet at 110–130 kDa were 73%, 61%, and 29%, respectively, of that seen in control cells treated with vanadate but in the absence of M1/89. These results indicate that inhibition of phosphorylation by M1/89 is due to its PTPase activity.

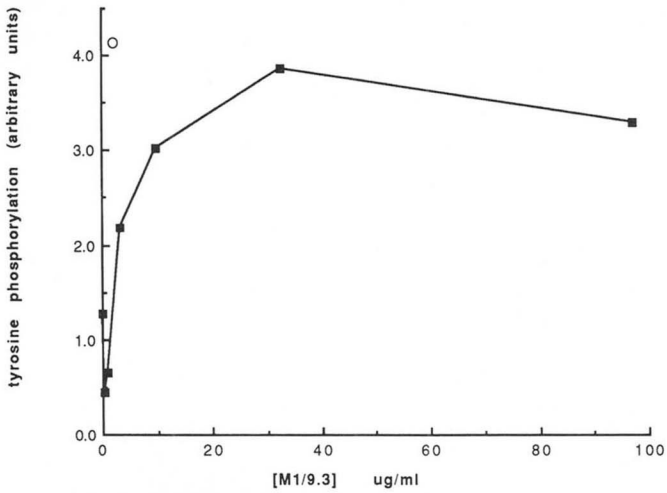

FIG. 8. **Influence of M1/9.3 on the inhibition of tyrosine phosphorylation of the 110–130-kDa doublet by M1/89.** Cells were incubated with 2 μg/ml M1/89 and various concentrations of M1/9.3 prior to activation with 500 ng/ml 2C11 (●) or 500 ng/ml 2C11 alone (○).

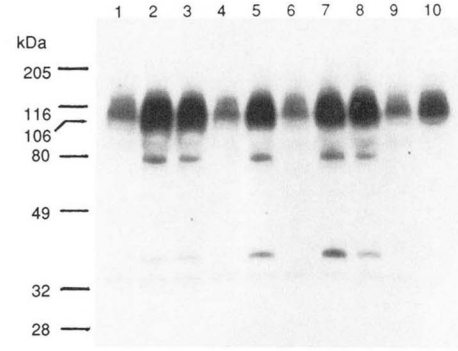

FIG. 9. **Dephosphorylation of phosphoproteins by M1/89 added after activation of the cells with 2C11.** Cells were activated with 500 ng/ml 2C11, and samples withdrawn and treated as described under "Materials and Methods." *Lanes 1–5*, 3 min after activation; *lanes 6–10*, 5 min after activation; *lanes 1* and *6*, no additions; *lanes 2* and *7*, + 500 ng/ml 2C11; *lanes 3* and *8*, pretreateated for 1 min with 2 μg/ml M1/9.3 prior to activation with 500 ng/ml 2C11; *lanes 4* and *9*, pretreated for 1 min with 2 μg/ml M1/89 prior to activation with 500 ng/ml 2C11; *lanes 5* and *10*, 2 μg/ml M1/89 added 4 min after activation with 500 ng/ml 2C11.

TABLE II

Influence of sodium orthovanadate on M1/89-stimulated PTPase activity

13.13 cells were preincubated with various concentrations of orthovanadate for 30 min at 37 °C prior to the addition of M1/89 (2 μg/ml) followed by 2C11 (500 ng/ml). Cells were harvested 5 min after the addition of the 2C11, and analyzed as described in the text. The phosphotyrosine content of the 110–130-kDa doublet was quantitated by densitometric scanning.

mAbs	Orthovanadate	Phosphotyrosine	
		Arbitrary units	%
	μM		
2C11	100	8.76	100
2C11 + M1/89	100	6.37	73
2C11 + M1/89	50	5.36	61
2C11 + M1/89	25	2.54	29

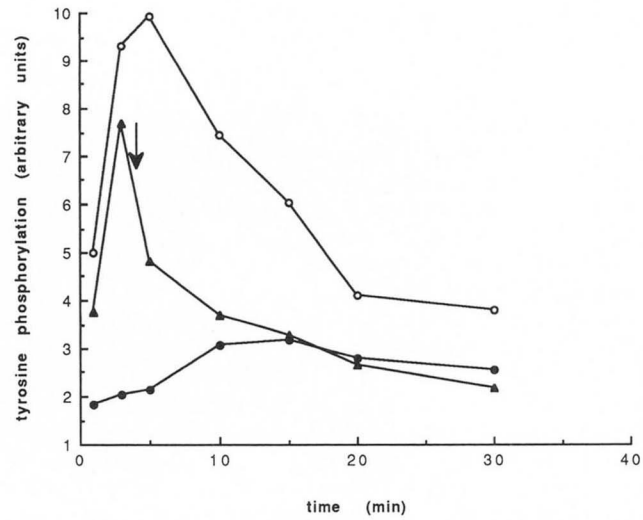

FIG. 10. **Kinetics of dephosphorylation of the 110–130-kDa doublet by M1/89.** Cells were treated as described in the legend to Fig. 9. ○, 2C11 alone; ●, pretreated with 2 μg/ml M1/89; ▲, 2 μg/ml M1/89 added at 4 min after activation with 500 ng/ml 2C11 (↓). The data set are from one experiment; similar results were obtained in two other experiments.

To determine whether M1/89 was inhibiting the initiation of activation of tyrosine phosphorylation stimulated by 2C11, or if the mAb was directly activating a PTPase which could subsequently dephosphorylate the proteins that became phosphorylated following activation of the cells with 2C11, the 13.13 cells were first activated with 2C11 and the M1/89 was subsequently added (Figs. 9 and 10). As previously observed, addition of 2C11 resulted in a 4–5-fold increase in tyrosine phosphorylation within 3 min (Fig. 9, compare *lane 1* with *lanes 2 and 3*); this increase was blocked by preincubation with M1/89 (Fig. 9, *lane 4*). When the M1/89 CD45 mAb was added to the cells 4 min after activation with 2C11, at which stage there was already a significant increase in tyrosine phosphorylation, there was a marked and rapid decrease in phosphotyrosine content of all of the bands (Fig. 9, *lane 10*). The kinetics of phosphorylation of the 110–130 kDa doublet in the presence of the various mAb combinations is shown in Fig. 10. Within 1 min of addition of the M1/89 mAb to the preactivated cells a marked decrease in the phosphorylation of the doublet was observed. This contrasted sharply with those cells treated with 2C11 alone which showed a further small increase in tyrosine phosphorylation between 3 and 5 min (Fig. 10). By 10 min the sample to which M1/89 was added 4 min post-activation, had levels of phosphotyrosine similar to those observed in cells that had been preincubated with the mAb, while the level of phosphorylation in cells treated with 2C11 alone remained markedly elevated. These results showed that even after activation of the cells with 2C11, M1/89 mAb is able to very rapidly activate a PTPase that is capable of dephosphorylating proteins that are phosphorylated upon cell activation.

DISCUSSION

Anti-CD45 mAbs have been found to influence significantly the activation of T-cells. This observation has lead to the suggestion that CD45 plays an important role in modulating T-cell responses. Anti-CD45 mAbs have been shown to enhance T-cell activation when simultaneously cross-linked using biotin/avidin, with mAbs to CD4 (8), inhibit T-cell activation when co-cross-linked with mAbs to CD3 (8, 4, 15), and either enhance or inhibit T-cell activation when co-cross-linked with mAbs to CD2 (8, 13, 14). Anti-CD45 mAbs that inhibited T-cell activation when coimmobilized with anti-CD3 on plastic are unable to inhibit activation induced by

anti-CD3 mAb when they were added in soluble form (15). Furthermore, activation induced by immobilized anti-CD3 is only partially inhibited by the independent binding of CD45 with a homoconjugate mAb (*i.e.* an antibody chemically cross-linked to itself) or by biotinylated anti-CD45 separately cross-linked with avidin (8, 15). Finally, activation of cells by heteroconjugates of anti-CD4 and anti-CD3 mAbs results in intracellular Ca^{2+} mobilization that is not inhibited by anti-CD45 mAb. Thus, binding of anti-CD45 mAbs to CD45 can result in enhancement or inhibition of T-cell responses or have little effect. The functional consequence of CD45 binding by mAbs appear to be dependent on the regimen of T-cell activation, the physical state of the antibodies, and the particular anti-CD45 mAb that is used. The molecular mechanisms responsible for these complex effects of anti-CD45 mAbs are, at present, unclear.

Functional effects of mAbs on T-cells often require either the presence of antigen-presenting cells or cross-linking by secondary reagents. In several experimental systems anti-CD45 have been shown to inhibit T-cell proliferation only when coimmobilized or cocross-linked with the activating mAbs, and there is a marked difference in responses if mAbs are are independently cross-linked (15). In the studies reported here both the CD3 and the M1/89 mAbs function in soluble form and without a requirement for cross-linking either mAb independently or cross-linking the mAbs together.

The cytoplasmic domain of CD45 has been shown to have intrinsic PTPase activity (3, 4). The mechanism by which this activity controls cell function, whether the activity is constitutive or inducible, and how the PTPase activity is directed toward its various protein substrates is unclear. Several potential substrates for CD45 PTPase have been identifed. These include $p56^{lck}$ (17, 18), a microtubule-associated protein-2 kinase (24), phospholipase C, and other as yet uncharacterized proteins (15). Reports that CD45 expression is essential for antigen-induced T-cell activation also suggest that the molecule may play a critical role in dephosphorylation of one or more proteins that are required for signaling through the TCR complex (10, 11).

We have analyzed the ability of two anti-CD45 mAbs to modulate T-cell activation in a murine T-cell hybridoma. One anti-CD45 mAb, M1/89, profoundly inhibited anti-CD3-stimulated IL-2 production and calcium mobilization, while the anti-CD45 mAb, M1/9.3, had little or no effect on either of these two parameters. The markedly different functional consequences of M1/9.3 and M1/89 on T-cell activation cannot be attributed to variations in the CD45 isoform that they recognize since the immunoprecipitation patterns were identical and each antibody was able to compete with the binding of the other. Furthermore, these functional distinctions also cannot be explained by a dissimilarity in the affinities of M1/9.3 and M1/89 for CD45, since antibody cross-blocking experiments indicate that these mAbs bind to the antigen with comparable affinity. Interestingly, the antibody cross-blocking experiments also show that M1/9.3 and M1/89 recognize epitopes that appear to be in close proximity. Finally, both M1/9.3 and M1/89-labeled CD45 were found to diffuse laterally in the cell membrane. These results suggest that it is unlikely that the functional differences between the two antibodies are a result of the ability of one but not the other to laterally immobilize CD45 and thereby alter the interaction of this molecule with potential substrates.

The effect of M1/89 mAb on anti-CD3-induced T-cell activation cannot be attributed to either a direct inhibition of binding of mAb to CD3, or to the M1/89 causing subsequent dissociation of antigen bound anti-CD3. Treatment of cells with M1/89 either before or after activation of the cells with 2C11 had no apparent effect on the binding of the anti-CD3 antibody over the time course of these experiments.

The inhibition of M1/89 mAb of anti-CD3-stimulated IL-2 production and Ca^{2+} mobilization suggests that CD3 and CD45 may associate functionally in the absence of coimmobilization. Consistent with this suggestion, a recent report (25) has shown that immunoprecipitation of TcR complex with the same anti-CD3 mAb used in this study results in coimmunoprecipitation of the CD45 molecule. The lack of an effect by M1/9.3 mAb in our system suggests that binding to a specific epitope on CD45 may be essential to stimulate activation of the phosphatase.

To address the basis of the distinct effects of the two anti-CD45 mAbs on IL-2 production and calcium mobilization, we compared the ability of the anti-CD45 mAbs to alter anti-CD3-stimulated protein tyrosine phosphorylation. T-cell activation by anti-TcR complex antibodies triggers several biochemical signal transduction pathways including phosphotidylinositol-specific phospholipase C activation, phosphotidylinositol hydrolysis and activation of tyrosine kinase (15), and microtubule-associated protein-2 kinase (24). Current evidence suggests that TcR-stimulated protein tyrosine phosphorylation may be a necessary prerequisite for mobilization of cellular Ca^{2+}, phospholipase C activation, and IL-2 production (26–29). Consistent with the observations of others, we show that anti-CD3 stimulation of the T-cell hybridomas leads to rapid tyrosine phosphorylation of several major bands and several other minor ones. The molecular masses of the four major phosphoproteins that we observed are 130, 110, 80, and 37 kDa. These molecular masses are similar to some of the substrates previously reported to be phosphorylated on tyrosines in activated T-cells or T-cell hybridomas (15, 26–29). Binding of CD45 by M1/9.3 caused little decrease in anti-CD3-stimulated protein tyrosine phosphorylation of these bands. In contrast, M1/89 mAb treatment dramatically reduced protein tyrosine phosphorylation. The ability of sodium orthovanadate, a PTPase inhibitor, to overcome significantly the effects of M1/89 binding to CD45 strongly suggests that the influence of CD45 is due to its PTPase activity. These effects of M1/89 on tyrosine phosphorylation, Ca^{2+} mobilization, and IL-2 production are consistent with other studies showing that inhibition of protein tyrosine phosphorylation can prevent IL-2 secretion and T-cell receptor-mediated cellular Ca^{2+} mobilization (26, 28, 29). To study further the mechanism by which M1/89 inhibits T-cell activation, the mAb was added to the cells several minutes after they had been activated by ligation of CD3. Immediately upon addition of M1/89 there was a marked decrease in the phosphotyrosine content of the four major protein substrates. This dephosphorylation occurred over a time period in which phosphorylation of the substrates in cells treated with anti-CD3 mAb alone was still increasing. The data indicate that CD45 can be rapidly activated to dephosphorylate the appropriate substrates and that binding to this molecule does not simply interfere with the ability of the CD3 complex to interact with or activate one or more tyrosine kinase. Recently Ledbetter and co-workers (15) have also found that cross-linking of CD45 after the initiation of T-cell activation influences the overall profile of cellular activation. In those experiments, which required cross-linking of mAbs, only a small effect on Ca^{2+} mobilization was observed.

The results reported here suggest that the differential effects of the anti-CD45 mAbs on protein tyrosine phosphorylation were due to their relative abilities to activate CD45 tyrosine phosphatase activity. Thus, M1/89 may recognize an

epitope on CD45 that is close to, or at, a site at which the putative ligand binds and is thereby able to induce the appropriate conformational changes that result in the activation of the PTPase. In contrast, although the M1/9.3 mAb appears to bind to an epitope on CD45 that is near that which M1/89 mAb binds, M1/9.3 is unable to stimulate the CD45 activity. The reason for this is presently unclear although it is likely that M1/9.3 mAb binding does not induce the conformational changes in CD45 that are required for PTPase activation.

Analogies can be made between the ability of different mAbs to activate CD45 PTPase and the different mAbs that bind to the EGF receptor. Several mAbs to the EGF receptor have been isolated and characterized (30, 31). One mAb, an IgM, mimics EGF in that it stimulates the tyrosine kinase activity and cellular DNA synthesis. If this antibody is used as a Fab fragment it retains its ability to activate the tyrosine kinase, but it does not stimulate receptor clustering and DNA synthesis unless it is cross-linked with a second antibody. A second mAb does not compete with EGF for binding to the receptor and does not induce the biological responses (30). A third group of mAbs are, in the intact cell, EGF antagonists. They block EGF binding and the *in vivo* stimulation of receptor phosphorylation (31). Thus, if the EGF receptor is taken as a model, it is not suprising that different mAbs to CD45 can induce different responses, some behaving as agonists and others having little effect. Furthermore, it is not unexpected that cross-linking of mAbs to CD45 can give rise to physiological responses that are more complex than seen using uncross-linked mAbs.

An advantage of the system that we have described here is that we have been able to stimulate T-cell activation and subsequently block it under conditions that have not required cross-linking or cocross-linking of either the stimulating or the blocking mAbs. This has allowed us to observe differential biological effects of two anti-CD45 mAbs that have similar binding affinities and, when complexed with antigen, have similar mobilities in the cell membrane. We have shown that CD45 function is dependent on its PTPase activity and that one of the anti-CD45 mAbs selectively activates the PTPase. This activating mAb may mimic the function of the putative ligand for CD45.

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