

# CD50 (Intercellular Adhesion Molecule 3) Stimulation Induces Calcium Mobilization and Tyrosine Phosphorylation through p59<sup>lck</sup> and p56<sup>lck</sup> in Jurkat T Cell Line

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## Summary

The leukocyte differentiation antigen, CD50, has been recently identified as the intercellular adhesion molecule 3 (ICAM-3), the third counter-receptor of leukocyte function-associated antigen 1 (LFA-1). This molecule seems to be specially involved in the adhesion events of the initial phases of the immune response. To characterize the role of CD50 in leukocyte interactions, the different molecular events induced after cross-linking of CD50 on T cell-derived Jurkat cell line have been analyzed. When cells were incubated with anti-CD50 mAbs and cross-linked with polyclonal goat anti-mouse immunoglobulins, a rise in intracellular calcium concentration ( $[Ca^{2+}]_i$ ) was observed. This increase in  $[Ca^{2+}]_i$  was mainly due to the uptake of extracellular  $Ca^{2+}$ . This  $Ca^{2+}$  flux involved tyrosine phosphorylations and was further increased by CD3 costimulation. These data, together with those obtained by phosphotyrosine (P-Tyr) immunoprecipitation and in vitro kinase assays, suggested the involvement of protein-tyrosine kinases (PTK) in CD50 transduction pathways. By using specific antisera, the presence of p56<sup>lck</sup> and p59<sup>lck</sup> protein tyrosine kinases (PTK) was clearly demonstrated in the CD50 immunoprecipitates. These findings suggest that the interaction of CD50 with its natural ligand (LFA-1) may result in T lymphocyte activation events, in which CD50 could play a very active role after antigen triggering.

The immune system, composed of recirculating cells, uses a dynamic and complex group of membrane molecules involved in cell-cell interaction mechanisms that are related at different degrees to antigen-dependent proliferation (1). These intercellular interactions are also especially relevant in several steps of inflammatory and immune responses. Among these steps, functions such as lymphocyte traffic between lymphoid organs and the bloodstream, lymphocyte migration to sites of injury, and antigen recognition are to be included (2). Intercellular adhesion is mediated by many different molecules, CD50 being a molecule that plays a prominent role in cell adhesiveness. This molecule has recently been identified by us as intercellular adhesion molecule 3 (ICAM-3)<sup>1</sup> (3), and it has been described as the third counter-receptor for the leukocyte integrin leukocyte function-associated antigen

1 (LFA-1) (4, 5). Moreover, CD50 seems to be a relevant molecule involved in leukocyte interactions (5, 6).

CD50 (ICAM-3) was described as a 120-kD, highly expressed, and hematopoietically restricted leukocyte surface antigen during the IV International Workshop on Human Leukocyte Differentiation Antigens (7), being originally defined by two mAbs (101-1D2 and 140-11) that were produced and characterized in our laboratory. The initial description of CD50 mAbs inhibiting allorecognition (6) suggested that CD50 could be a relevant molecule involved in leukocyte interactions. The finding that CD50 became rapidly and transiently phosphorylated on serine residues (8) upon stimulation either by PMA or T cell receptor cross-linking suggested a biochemical regulation of its function. The analysis of CD50 cDNA clones (9-11) showed the highest homology with ICAM-1 and ICAM-2, especially in their extracellular domains. However, comparison of the intracellular regions showed a low similarity, suggesting a different function for the intracytoplasmic domain of each different ICAM molecule (9, 11). Indeed, five serine residues in CD50 cytoplasmic region are, probably, the residues that become phosphorylated

<sup>1</sup> Abbreviations used in this paper:  $[Ca^{2+}]_i$ , intracellular calcium concentration; GAM, goat anti-mouse immunoglobulins; ICAM, intercellular adhesion molecule; IP<sub>3</sub>, D-myo-inositol 1,4,5-trisphosphate; LFA-1, leukocyte-associated antigen 1; PKC, protein kinase C; PTK, protein tyrosine kinase.

in CD50 molecule after protein kinase C (PKC) stimulation induced by PMA or CD3 (8). This phosphorylation could be a way of controlling a possible CD50 function, such as the LFA-1 phosphorylation in response to phorbol ester (12). Unlike its homologous ICAM-1 and ICAM-2, this new member of the Ig gene superfamily is highly expressed on resting lymphocytes. This fact strongly suggested that CD50 is preferentially used by lymphocytes in the LFA-1-mediated binding during the initial interactions of immune recognition and lymphocyte activation (5, 9). Furthermore, its lack of expression in endothelial cells (9, 10) leads us to assume that its possible role in the leukocyte-endothelial cell is indirect.

It has been repeatedly described that, in the ICAM-1/LFA-1 interaction, the LFA-1 plays the most important role in signal transduction (13-17). This signaling pathway through LFA-1 involves calcium mobilization, phosphoinositide hydrolysis, amplification of CD3-dependent IL-2 production, and proliferation and enhancing antigen presentation in B cells. Whereas direct intracellular signaling pathways through LFA-1 have been extensively described, there are few data about transducing effects through ICAM-1 (18, 19) and ICAM-2 (20). All these data suggested that the main functional role of both ICAM-1 and ICAM-2 was their binding with LFA-1 which triggered some transduction signals that affected directly other stimulatory surface proteins, enhancing cell activation. If one considers the high homology of CD50 with ICAM-1 and ICAM-2, and the fact that little is known about the functional involvement of CD50 in cell activation it could easily be assumed that CD50, like ICAM-1 and ICAM-2, would play only a secondary role in signal transduction. However, since its first description (3), the important differences, described above (cell distribution and cell induction, intracytoplasmic domain, phosphorylation by PKC stimulation), suggested that CD50 could play a more active role in signal transduction than ICAM-1 or ICAM-2.

To analyze the role of CD50 in immune interactions, the ability of anti-CD50 mAbs to transduce signals into Jurkat cells, a T-derived cell line, was studied. In this study, direct biochemical and cellular evidences are described showing that CD50 (ICAM-3) is a molecule capable of transducing signals across the plasma membrane of these cells. More precisely, our results show a direct relationship between two protein tyrosine kinases (PTKs), p56<sup>lck</sup> and p59<sup>lyn</sup> (two nonreceptor PTKs of the src family), and CD50 molecule, suggesting their involvement in the CD50 signaling pathway. These data contribute to a better understanding of CD50 (ICAM-3) activation pathways and present CD50 as a new costimulatory molecule with a probable enhancing role in immune response.

## Materials and Methods

**Antibodies and Cell Line.** The following murine mAb to human antigens were used at the indicated concentrations: 101-1D2 (anti-CD50, IgG1) (6); 140-11 (anti-CD50, IgG2b) (6); 152-2D11 (anti-CD50, IgG1) (3); 143-14 (CD27, IgG2a) (21); RM3A5 (anti-CD54, IgG2a) (22); 72-5D3 (anti-CD45, IgG1) (23); Cris-7 (anti-CD3, IgG1) (23); 124-1D1 (anti-CD7, IgG1) (24); 133-1G6 (IgM) (25); and nonbinding NS1 ascites as a control (IgG1). All antibodies were

purified by protein A affinity chromatography. Second step polyclonal goat anti-mouse Ig (GAM) (affinity purified) was purchased from Tago, Inc. (Burlingame, CA). P-Tyr (Ab-1), anti-phosphotyrosine (P-Tyr) mAb was obtained from Oncogene Science, Inc. (Manhasset, NY). Rabbit polyclonal antiserum to p56<sup>lck</sup> (26) and p59<sup>lyn</sup> (27) were kindly provided by Dr. R. M. Perlmutter (University of Washington, Seattle, WA). The Jurkat T-derived lymphoblastoid cell line was obtained from the European Collection of Cultured Cell Lines (Salisbury, UK) and was maintained in culture in RPMI 1640 medium supplemented with 10% FCS.

**[Ca<sup>2+</sup>]<sub>i</sub> Measurements.** INDO 1-AM cell labeling was performed according to a standard protocol (28). Briefly, Jurkat cells were washed twice in INDO 1 buffer A (HBSS medium with 10 mM Hepes, pH 7.0), counted, and resuspended at 5 × 10<sup>6</sup> cells/ml. The fluorescent Ca<sup>2+</sup> indicator INDO 1-AM (Molecular Probes, Inc., Eugene, OR) was added to cell lines to 12.5 μM. After a 30-min incubation at 37°C, the same volume of INDO 1 buffer B (HBSS with 10 mM Hepes and 5% FCS, pH 7.4) was added and reincubated for 30 min at 37°C. Then, cells were washed twice with INDO 1 buffer C (HBSS with 10 mM Hepes, 10 μM DNAase, and 5% FCS, pH 7.2), counted, and resuspended at 5 × 10<sup>6</sup> cells/ml at room temperature. Several minutes before the stimulation, cells were resuspended at 5 × 10<sup>5</sup> cells/ml in INDO 1 buffer C and maintained at 37°C. Cells were incubated with the different mAb for 15 min, and then washed twice. Cross-linking stimulation of mAbs was made with a second step polyclonal GAM, during the analysis. In some experiments CaCl<sub>2</sub> was omitted from the INDO 1 buffer C medium, and EGTA 100 μM added instead. After the complete response without extracellular Ca<sup>2+</sup>, 10 μM CaCl<sub>2</sub> was added to the medium. Both Genistein and Herbimycin A (both from Calbiochem, La Jolla, CA) were used as PTK inhibitors. In the experiments where Genistein was used, this inhibitor was added 30 min before the analysis at 150, 75, 25, and 7.5 μM. In the experiments where Herbimycin A was used, cells were preincubated with this PTK inhibitor 16 h before the assay. Both inhibitors were maintained throughout the assay. Fluorescent measurements were carried out on a FACStar-plus<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA), using an argon ion laser, focused in the UV (351-363 nm). INDO 1-AM emissions were detected at 405/22 and 485/22 nm and analyzed by Chronos software (Becton Dickinson & Co.) a software package that allows the evaluation of the mean INDO 1-AM violet/blue fluorescence ratio vs. time.

**D-myo-Inositol 1,4,5 Trisphosphate (IP<sub>3</sub>) Determination.** Jurkat cells on HBSS medium (BSS + 50 mM Hepes + 5% FCS) were treated with 50 μg/ml of anti-CD50 (152-2D11), 1 ng/ml of anti-CD3 (positive control), or GAM alone (negative control), for 20 min at 4°C. Excess antibodies were removed by centrifugation and pellets were suspended in 1 ml of HBSS medium with or without 20 μg/ml of GAM. Incubation was carried out for the indicated time and stopped by addition of 200 μl ice-cold perchloric acid 20%. After a 20-min incubation on ice, samples were centrifuged (2,000 g) and supernatants were titrated to pH 7.5 with 1.5 M KOH and 60 mM Hepes buffer. IP<sub>3</sub> levels were measured with IP<sub>3</sub> [<sup>3</sup>H] assay system (Amersham International, Little Chalfort, UK) by the competition between tested unlabeled IP<sub>3</sub> and a fixed quantity of [<sup>3</sup>H]IP<sub>3</sub> for a bovine adrenal binding protein with a limited number of binding sites.

**<sup>32</sup>P-Orthophosphate Radiolabeling and P-Tyr Immunoprecipitation Analysis.** After washing twice in phosphate-free medium (20 mM Hepes, 10 mM dextrose, 1 mM CaCl<sub>2</sub>, 5 mM KCl, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, and 20 mM NaHCO<sub>3</sub>, pH 7.2), 2 × 10<sup>6</sup> cells/ml were suspended in this phosphate-free medium supplemented with 10% dialyzed FCS containing 500

$\mu\text{Ci/ml}$  of [ $^{32}\text{P}$ ]orthophosphate (PBS 11; Amersham International). Radiolabeling was carried out for 3 h in 6-well tissue culture plates (Costar Corp., Cambridge, MA) at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  according to a standard protocol (29). Once labeled, cells were incubated for 30 min with each mAb. Excess antibodies were removed by centrifugation and pellets were resuspended in 1 ml of medium with 25  $\mu\text{g/ml}$  of GAM. Incubation was carried out for 5 min and then, cells were disrupted at  $4^\circ\text{C}$  for 30 min in a lysis buffer containing protease and phosphatase inhibitors (30). After four clearing cycles with an unrelated mAb plus protein A-Sepharose, supernatants were adjusted to contain identical acid-insoluble radioactivity and incubated for 1–2 h with 10  $\mu\text{l}$  of P-Tyr (Ab-1) anti-P-Tyr mAb. Then, lysates were incubated for 1–2 h with 50  $\mu\text{l}$  protein A-Sepharose packed beads adsorbed with a rabbit anti-mouse Ig serum (Dako, Glostrup, Denmark). The resulting immune complexes were washed, eluted, and analyzed by SDS-PAGE (31) in 10% polyacrylamide slab gels at 30 mA/gel. The gel was dried and exposed to Kodak X-Omat AR films with intensifying screens at  $-80^\circ\text{C}$ . For assessing molecular masses, a standard  $^{14}\text{C}$ -labeled protein mixture (Amersham International) was included in the gels.

**In Vitro Kinase Assay.** Solubilization and kinase reactions were performed as previously described (32), with some modifications. Briefly, cells were harvested and solubilized at  $2 \times 10^7$  cells/ml in either 1% Triton X-100 lysis buffer (1% Triton X-100/150 mM NaCl with 1 mM sodium orthovanadate + 1 mM EDTA + leupeptin [10 mg/ml] + aprotinin [10 mg/ml] + 25 mM *p*-nitrophenyl-*p*-guanidinobenzoate) or in 1% digitonin lysis buffer (1% digitonin/150 mM NaCl/50 mM Hepes, pH 7.5, with the above inhibitors; digitonin was prepared as described by Oettgen et al. [33]). Soluble lysates from  $10^7$  cells were incubated at  $4^\circ\text{C}$  for 1 h with different mAb and then, immunoprecipitated for 1 h with protein A-Sepharose (Pharmacia LKB, Uppsala, Sweden). After immunoprecipitation, immune complexes were washed in lysis buffer with appropriate detergent but without EDTA. In some cases, 1 M  $\text{MgCl}_2$  lysis buffer was used for washing out associated molecules (3). Immunocomplex kinase assays were performed by incubation at  $4^\circ\text{C}$  for 15 min in 50  $\mu\text{l}$  kinase buffer (150 mM NaCl + 20 mM Hepes, pH 7.5, + 5 mM  $\text{MnCl}_2$  + 5 mM  $\text{MgCl}_2$  + 1 mM ATP + 10 mCi of  $\gamma$ -[ $^{32}\text{P}$ ]ATP). The reaction was stopped with lysis buffer containing the appropriate detergent and 20 mM EDTA. Immunocomplexes were washed twice in lysis buffer, eluted in SDS sample buffer and analyzed by SDS-PAGE 8% (30).

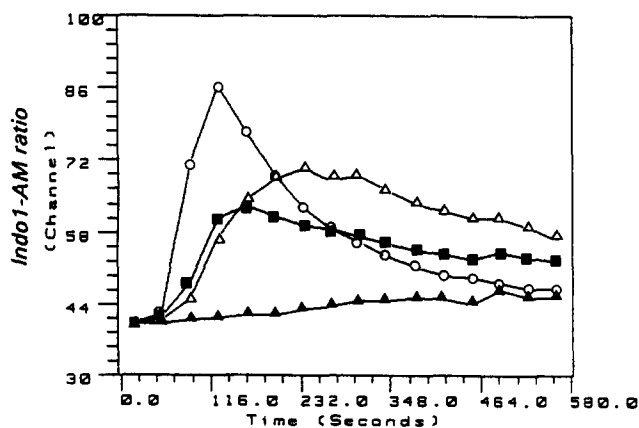
**Phosphoamino Acid Analysis.**  $^{32}\text{P}$ -labeled proteins were electroeluted from excised bands of polyacrylamide gels, precipitated with cold acetone/ $\text{NH}_3$  (5.3:0.3) and hydrolyzed in 0.2 ml 6 M HCl at  $110^\circ\text{C}$  for 2 h. Acid-hydrolyzed samples were dried, resuspended in 0.2 ml  $\text{H}_2\text{O}$ , and dried again. The residue was then resuspended in 10  $\mu\text{l}$  in electrophoresis buffer (pyridine/acetic acid/water, 5:50:945), pH 3.5. Samples of 2–10  $\mu\text{l}$  were spotted on cellulose-coated plates (E. Merck, Darmstadt, Germany), next to 1  $\mu\text{l}$  of a phosphoamino acid standard solution (phosphoserine, phosphothreonine, and phosphotyrosine, milligram per milliliter each), and analyzed by one-dimensional thin-layer electrophoresis (1000 V, 45 min). Nonradioactive standards were detected with 0.25% ninhydrin in ethanol and radiolabelled phosphorylated amino acids by autoradiography.

**CD50 Immunoprecipitation and PTK (Western) Blot Analysis.** To control the efficiency of immunoprecipitation, we previously labeled cells with  $^{125}\text{I}$ . For this radiolabeling, Jurkat cells were surface-labeled with  $^{125}\text{I}$  using Iodogen (Pierce Chemical Co., Rockford, IL) (34). After labeling,  $2 \times 10^7$  Jurkat cells were lysed in 1 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1 mM NaF, 1 mM PMSF,

20  $\mu\text{l/ml}$  egg white trypsin inhibitor, 1  $\mu\text{g/ml}$  leupeptin, and 1  $\mu\text{g/ml}$  pepstatin) for 30 min on ice. Nuclei and insoluble debris were removed by centrifugation at 14,000  $g$  for 10 min and lysates precleared with protein A-Sepharose (Pharmacia LKB, Uppsala, Sweden), prewashed in lysis buffer. Immunoprecipitation was performed with the indicated mAb, coupled to CNBr-activated Sepharose CL-4B (Pharmacia LKB) for 1 h at  $4^\circ\text{C}$ , washed six times with washing buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.05% NP-40, 0.05% SDS, and with the above inhibitors) and eluted with  $2\times$  SDS reducing sample buffer. Samples were counted with a  $\gamma$  counter. The same quantity of cpm for each sample was loaded in each lane and samples were run on 8% SDS-PAGE (31), and transferred by electroblotting (4 mA/cm $^2$ ) with glycine transfer buffer (39 mM glycine, 48 mM Tris-base, 1.3 mM SDS, and 20% methanol) to nitrocellulose. SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were used. After incubating filters with blocking solution (5% ovalbumin [Sigma Chemical Co., St. Louis, MO] and 3% nonfat milk protein in PBS) overnight at  $4^\circ\text{C}$ , blots were incubated with anti-p56 $^{lck}$  and anti-p59 $^{src}$  diluted to 1:1,000 in blocking solution for 1 h at  $20^\circ\text{C}$ . After washing with 0.25% gelatin in PBS, filters were incubated with goat anti-rabbit coupled to peroxidase in blocking solution for 1 h at  $20^\circ\text{C}$ . Filters were washed again with 0.25% gelatin in PBS, and the Western blots were developed using Amersham's enhanced chemiluminescence (ECL) reagent (Amersham International).

## Results

**CD50 (ICAM-3) Cross-linking Induces a Transient Increase in Intracellular Calcium Levels of Jurkat Cells.** The ability of three different anti-CD50 mAbs (101-1D2, 152-2D11, and 140-11) to induce a  $\text{Ca}^{2+}$  mobilization across the membrane of Jurkat cells was analyzed. It has been previously shown that these three mAbs recognize different epitopes of the CD50 (ICAM-3) (3). The results in Fig. 1 show clearly that two of them, namely 101-1D2 and 152-2D11, are able to induce a transient increase in  $[\text{Ca}^{2+}]_i$  by their binding and cross-linking to Jurkat cells, whereas 140-11 does not induce any change. The  $\text{Ca}^{2+}$  mobilization obtained with the 101-1D2



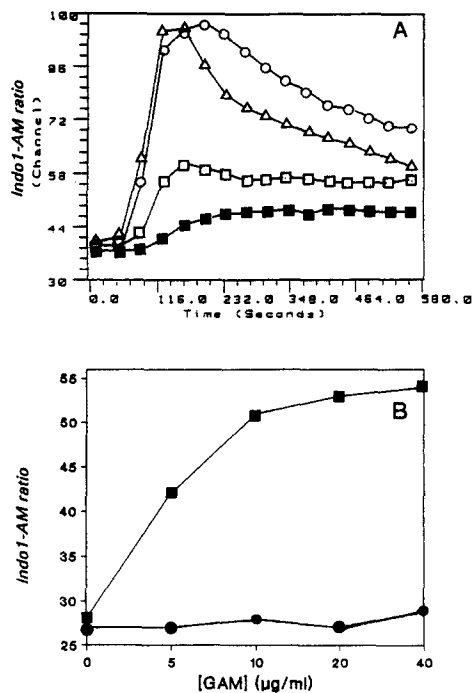
**Figure 1.**  $\text{Ca}^{2+}$  mobilization after CD50 cross-linking. Effect of anti-CD50: Cris 7, 1 ng/ml (O-O); and three anti-CD50s, 50  $\mu\text{g/ml}$ : 152-2D11 ( $\Delta$ - $\Delta$ ); 101-1D2 ( $\blacksquare$ - $\blacksquare$ ); 140-11 ( $\blacktriangle$ - $\blacktriangle$ ); cross-linked at 50 s with 10  $\mu\text{g/ml}$  of GAM.

mAb has always been identical or lower than the one observed with the 152-2D11 mAb. The lack of response obtained with the 140-11 mAb, even at the highest dose tested (100  $\mu\text{g}/\text{ml}$ ), confirms that this mAb and 152-2D11 and 101-1D2 mAbs recognize different functional epitopes of CD50 molecule. As negative controls for calcium mobilization, several mAbs (RM3A5, 124-1D1, and 133-1G6) of different subtypes (IgG2a, IgG1, and IgM, respectively) recognizing different Jurkat membrane antigens have been used. Whereas the binding and cross-linking of these mAb did not induce any detectable  $[\text{Ca}^{2+}]_i$  increase, the cross-linking of CD3 has been used as positive control for  $\text{Ca}^{2+}$  mobilization in all the tested situations (data not shown). The transient increase in  $[\text{Ca}^{2+}]_i$  obtained by CD50 cross-linking is similar to the effect observed by cross-linking very low doses of anti-CD3 mAb (1 ng/ml) (Fig. 1) (at a higher dose  $\geq 10$  ng/ml), anti-CD3 mAb is able to induce an increase in  $[\text{Ca}^{2+}]_i$  without cross-linking by GAM). A singular characteristic of the effect obtained with the CD50 mAb was the fact that although the maximum effect of CD50 cross-linking took longer to be reached than the one obtained by CD3 mAb, the increase of  $[\text{Ca}^{2+}]_i$  observed using CD50 mAbs reached a higher and more sustained plateau than the plateau obtained with CD3 mAb: CD50 effect reached its maximum level at 100–120 s after stimulation (70–100 s with CD3), but it was maintained at a high plateau (higher than CD3 stimulation) for longer than 500 s.

Dose-response experiments were performed using different concentrations of anti-CD50 mAb (Fig. 2 A) and cross-linking antibody (Fig. 2 B). The transient increase  $[\text{Ca}^{2+}]_i$  was shown to be proportional to the amount of anti-CD50 mAb. A minimum effect was observed with a dose as low as 2  $\mu\text{g}/\text{ml}$  and the maximum level was reached with oversaturating amounts of mAbs (100  $\mu\text{g}/\text{ml}$ ) (Fig. 2 A). Regarding the cross-linking antiserum, the relative increase in  $[\text{Ca}^{2+}]_i$  was proportional to the concentration used, reaching its highest level with oversaturating (50  $\mu\text{g}/\text{ml}$ ) amounts of GAM (Fig. 2 B). Taking all these data into account, the doses of 50  $\mu\text{g}/\text{ml}$  of 152-2D11 and 10  $\mu\text{g}/\text{ml}$  of GAM, which were shown to be clearly stimulating, were chosen as standard doses for all subsequent assays.

**Simultaneous CD3 and CD50 (ICAM-3) Cross-linking; Involvement of Different  $\text{Ca}^{2+}$  Sources.** When Jurkat cells were treated simultaneously with stimulating concentrations of both CD3 and CD50 mAb, plus GAM, a high increase in  $[\text{Ca}^{2+}]_i$  was observed (Fig. 3 A). The increase in  $[\text{Ca}^{2+}]_i$  was sharper than the one observed when CD3 or CD50 mAbs were used alone, whereas the peak of  $[\text{Ca}^{2+}]_i$  reached under these conditions was similar to the addition of the effects of anti-CD50 and anti-CD3 used separately. Moreover, the increase in  $[\text{Ca}^{2+}]_i$  using both mAbs simultaneously was prolonged on a level higher than the one obtained using CD3 or CD50 mAb alone. Therefore, the response to both mAb seems to be the addition of each separated response, resulting in a global improvement of the  $[\text{Ca}^{2+}]_i$  signaling.

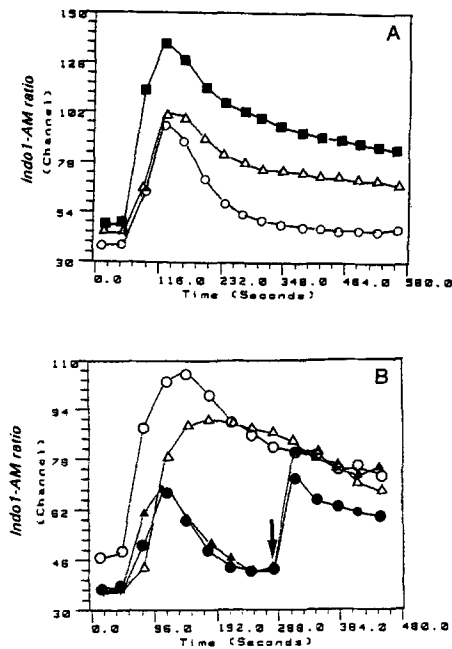
To better characterize the mechanisms responsible for the effect of anti-CD50 mAb,  $\text{Ca}^{2+}$  mobilization in Jurkat cells



**Figure 2.** Dose-response analysis on  $\text{Ca}^{2+}$  mobilization after CD50 cross-linking. (A) Calcium increases at different concentrations of 152-2D11: 100 (O-O), 50 ( $\Delta$ - $\Delta$ ), 10 ( $\square$ - $\square$ ), and 2 ( $\blacksquare$ - $\blacksquare$ )  $\mu\text{g}/\text{ml}$ , cross-linked at 50 s with 10  $\mu\text{g}/\text{ml}$  of GAM. (B) INDO 1-AM levels after stimulation with 50  $\mu\text{g}/\text{ml}$  of 152-2D11 with different doses of GAM, at 0 s ( $\bullet$ - $\bullet$ ) and 120 s ( $\blacksquare$ - $\blacksquare$ ) after cross-linking.

was analyzed in  $\text{Ca}^{2+}$ -free, EGTA-containing medium before and after the  $\text{CaCl}_2$  addition (Fig. 3 B). Under  $\text{Ca}^{2+}$ -free conditions, a rapid increase could be observed as a result of  $\text{Ca}^{2+}$  redistribution from intracellular stores; however, this effect was shorter and smaller than the increase observed in medium with  $\text{Ca}^{2+}$ . The characteristic sustained plateau that is observed stimulating with CD50 mAb and which is higher than the level obtained upon CD3 stimulation, disappeared using  $\text{Ca}^{2+}$ -free conditions. Subsequent addition (Fig. 3 B, arrow) of  $\text{Ca}^{2+}$  to the medium (10  $\mu\text{M}$   $\text{CaCl}_2$ ) induced a further  $[\text{Ca}^{2+}]_i$  increase, as a result of the uptake of extracellular  $\text{Ca}^{2+}$ . This increase achieved a plateau as high as the one obtained using the medium with  $\text{Ca}^{2+}$ . All these data suggest that the increase in  $\text{Ca}^{2+}$  obtained by anti-CD50 stimulation is mainly due to the uptake of extracellular  $\text{Ca}^{2+}$ . This extracellular  $\text{Ca}^{2+}$  would be responsible for the characteristic prolongation of the increase, whereby the  $\text{Ca}^{2+}$  from the internal stores would be responsible only for the initial increase, and even in this case in a minor way.

To analyze the role of inositol phospholipid hydrolysis in CD50  $\text{Ca}^{2+}$  mobilization, the  $\text{IP}_3$  levels on stimulated Jurkat cells were evaluated. After CD50 stimulation with 152-2D11 cross-linking, a slight but clear increase of  $\text{IP}_3$  at 15 s (Table 1) was detected;  $\text{IP}_3$  increase induced by CD50 mAb was lower than the increase induced by CD3 mAb used as positive control. No  $\text{IP}_3$  increase was observed by using GAM



**Figure 3.**  $Ca^{2+}$  mobilization after CD3 and CD50 co-cross-linking, and after CD50 and CD3 individual cross-linking, in absence of extracellular  $Ca^{2+}$ . (A) Additional effect of the costimulation with anti-CD3 (Cris-7) and anti-CD50 on Jurkat cell line. Cris-7 (O-O), 1 ng/ml; 152-2D11 ( $\Delta$ - $\Delta$ ), 50  $\mu$ g/ml; and 152-2D11 + Cris-7 ( $\blacksquare$ - $\blacksquare$ ), 50  $\mu$ g/ml + 1 ng/ml; cross-linked at 50 s with 10  $\mu$ g/ml of GAM. (B) Jurkat cells were stimulated by cross-linking CD3 and CD50 at 50 s in an INDO-1 buffer C with 100 mM EGTA and without  $CaCl_2$  and or with 10  $\mu$ M  $CaCl_2$  was added at 300 s (arrow). Stimulations in presence of extracellular  $Ca^{2+}$  are also presented. Cris-7, 1 ng/ml, with (O-O) and without ( $\bullet$ - $\bullet$ ) extracellular  $Ca^{2+}$ , and 152-2D11, 50  $\mu$ g/ml, with ( $\Delta$ - $\Delta$ ) and without ( $\blacktriangle$ - $\blacktriangle$ ) extracellular  $Ca^{2+}$ , cross-linked at 50 s with 10  $\mu$ g/ml of GAM.

alone (negative control). Thus, the effect of CD50 stimulation on  $IP_3$  levels correlated perfectly with the initial induction of  $Ca^{2+}$  mobilization. This correlation could probably explain the intracellular component of  $Ca^{2+}$  increase.

**$Ca^{2+}$  Mobilization Induced by CD50 (ICAM-3) Cross-linking Is Mediated by Tyrosine Phosphorylation.** To further analyze the signal transduction pathway responsible for the effect of CD50 mAb, the role of tyrosine phosphorylation on the increase in  $[Ca^{2+}]_i$  (Fig. 4) was studied. Pretreatment of Jurkat

cells for 30 min and throughout the assay with 7.5, 25, 75, and 150  $\mu$ M of Genistein showed an inhibitory effect on  $Ca^{2+}$  mobilization. This inhibition was partial at 7.5, 25, and 75  $\mu$ M and almost total at 150  $\mu$ M (Fig. 4 A). Instead, the stimulatory effect of CD3 was only partially inhibited by Genistein (data not shown), blunting the  $Ca^{2+}$  increase, as previously described (35). Furthermore, pretreatment of Jurkat cells for 16 h with Herbimycin A at 1  $\mu$ M also completely prevented the increase in  $[Ca^{2+}]_i$  induced by CD50 mAbs (Fig. 4 B). As previously described (36), Herbimycin A also totally inhibited CD3  $Ca^{2+}$  mobilization, although an increase in  $[Ca^{2+}]_i$  induced by A23187 ionophore was completely resistant to both Genistein and Herbimycin A (data not shown).

For a better understanding of the role of PTK in  $Ca^{2+}$  mobilization induced by CD50 mAb, the effect of CD45 on this effect was analyzed. A mAb against CD45, which is a molecule with phosphatase activity (37), was incubated simultaneously with CD50 mAb. A total inhibition on the CD50  $Ca^{2+}$  increase was observed with the co-cross-linking of CD50 and CD45 mAb (Fig. 4 C). These data are in agreement with similar observations where phosphatase activity of CD45 was shown to inhibit analogous  $Ca^{2+}$  mobilizations dependent on kinase activity (38, 39).

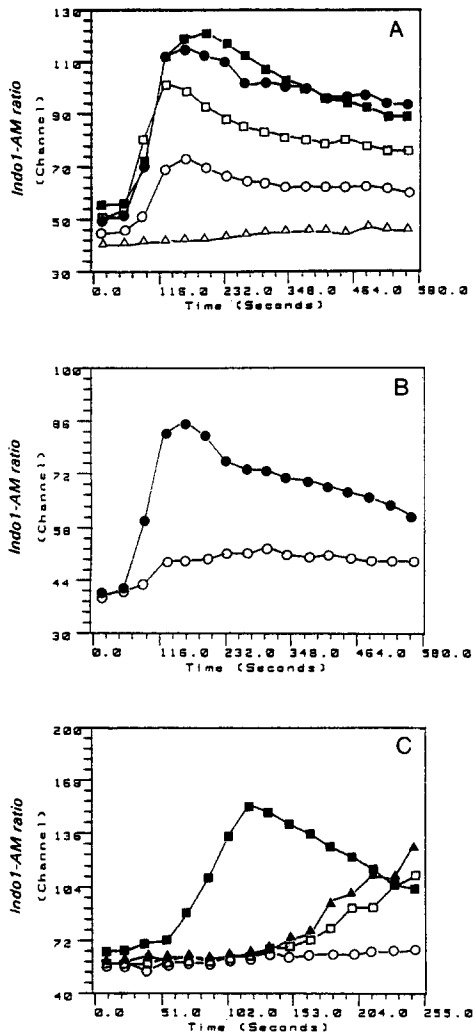
**CD50 (ICAM-3) Cross-linking Induces Tyrosine-Phosphorylation of 33- and 40-kD-specific Substrates.** The effects of both, PTK inhibitors and CD45 on CD50 induced  $Ca^{2+}$  mobilization made us analyze the pattern of tyrosine phosphorylation after CD50 cross-linking.  $P^{32}$ -labeled Jurkat cells (Fig. 5) were treated with CD50 mAb (152-2D11) and cross-linked with GAM for 5 min. After immunoprecipitation with an anti-P-Tyr mAb of the lysates, a remarkable increase in the tyrosine phosphorylation pattern was observed. Especially relevant was the effect on a 33-kD molecule that appeared phosphorylated after CD3 and CD50 stimulation and was absent in the negative control. In addition, a significant increase in the phosphorylation of a 40-kD protein after CD50 cross-linking was also detected.

**CD50 (ICAM-3) mAb Coimmunoprecipitates an Associated Tyrosine Kinase Activity: both  $p56^{lck}$  and  $p59^{fyn}$  PTK Are Detected in Association with CD50.** The immunoprecipitate obtained with the three CD50 mAb were tested in an in vitro kinase assay. Fig. 6 A shows the pattern of phosphorylation

**Table 1.**  $IP_3$  Mobilization after Cross-linking of CD50 Stimulation (pmols/ $10^6$  Cells)

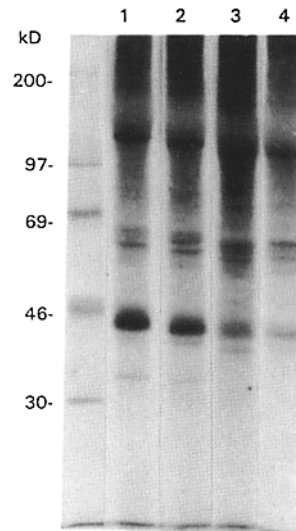
	0 s	15 s	30 s	1 min	2 min	5 min	10 min	15 min
GAM	0.39	0.23	0.42	0.22	0.20	0.45	0.42	0.19
CD50	0.23	1.00	0.57	0.50	0.62	0.42	0.40	0.22
CD3	0.40	1.20	0.75	0.58	0.50	0.45	0.39	0.41

Jurkat cells were incubated on HBSS medium with 50  $\mu$ g/ml of anti-CD50 (152-2D11), 1 ng/ml of anti-CD3 (positive control), and GAM alone (negative control), for 20 min at 4°C. Excess antibodies were removed and cells were cross-linked in 1 ml of HBSS medium with 25  $\mu$ g/ml of GAM. Incubation was carried out for the indicated times and stopped by addition of 200  $\mu$ l ice-cold perchloric acid 20%. Samples were centrifuged and  $IP_3$  levels of the supernatants were measured with  $IP_3$   $^3H$  assay system (Amersham International).



**Figure 4.**  $\text{Ca}^{2+}$  mobilization induced after CD50 stimulation is inhibited by Genistein and Herbimycin A treatment and after co-cross-linking of CD50 and CD45. (A) Inhibition effect of Genistein at 0 (■-■), 7.5 (●-●), 25 (□-□), 75 (○-○), and 150 (△-△)  $\mu\text{M}$  in CD50 stimulation. (B) Inhibition effect of Herbimycin A at 0 (●-●) and 1 (○-○)  $\mu\text{M}$ . (C) Inhibition effect of co-cross-linking with anti-CD45 (72-5D3) and anti-CD50 (152-2D11), 152-2D11 (■-■), 50  $\mu\text{g}/\text{ml}$ ; 72-5D3 (□-□), 50  $\mu\text{g}/\text{ml}$ ; 152-2D11 + 72-5D3 (▲-▲), 50  $\mu\text{g}/\text{ml}$  + 50  $\mu\text{g}/\text{ml}$ ; cross-linked at 50 s with 10  $\mu\text{g}/\text{ml}$  of GAM and (○-○) only GAM.

obtained when 1% Triton X-100 was used as detergent. A specific phosphorylation of a 31-kD molecule was observed, confirming the presence of a kinase activity associated with the CD50 molecule, contrasting with the lack of phosphorylation after immunoprecipitating with a negative control mAb (anti-CD27). In addition, we had previously shown that washing in 1 M  $\text{MgCl}_2$  is able to eliminate all the associated molecules without eluting CD50 molecule from the mAb binding (3). The results in Fig. 6 A show that the kinase activity was eluted by using 1 M  $\text{MgCl}_2$  washing buffer, suggesting that this kinase activity was not intrinsically due to the CD50 molecule. To assess the type of phosphorylation, the 31-kD phosphorylated protein was hydrolyzed and



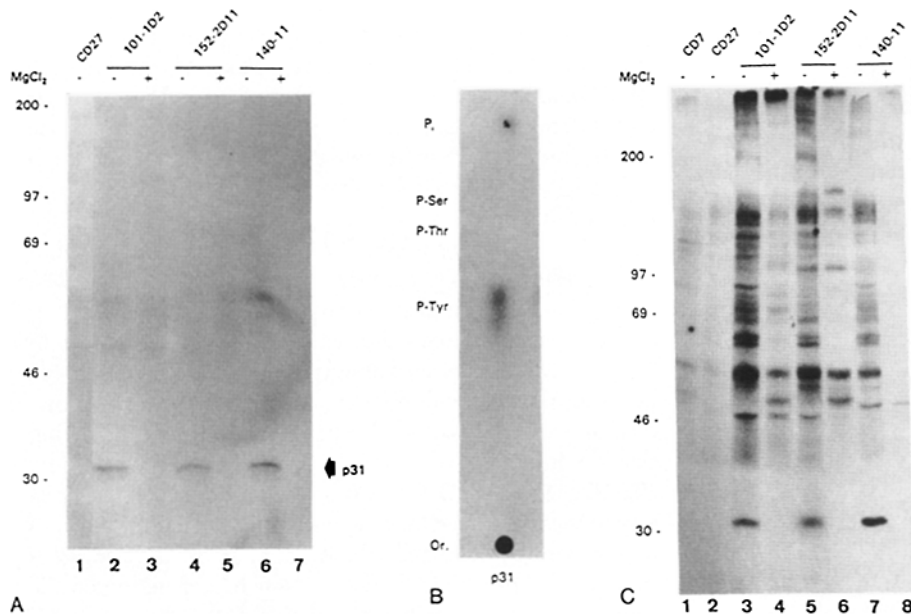
**Figure 5.** Immunoprecipitation of Jurkat cells lysates stimulated by CD50, with anti-P-Tyr. Jurkat cells were incubated with [ $^{32}\text{P}$ ]orthophosphate for 3 h and stimulated by 152-2D11, CD50 (lane 1), Cris-7, CD3 (lane 2) and PMA (lane 3). Lane 4 shows basal phosphorylation without stimulation. CD50 cross-linking induces tyrosine-phosphorylation of 33- and 40-kD-specific substrates (arrows).

subjected to phosphoamino acid analysis which showed a phosphorylation on tyrosine residues (Fig. 6 B). To further confirm these results, an in vitro kinase assay with 1% digitonin buffer was performed. This procedure produced a stronger and more complex pattern of phosphorylation, which also included the 31-kD molecule. The kinase activity was also washed out by using 1 M  $\text{MgCl}_2$  solution (Fig. 6 C), thus confirming the previous observation. Negative controls with mAb against unrelated Jurkat molecules (CD7 and CD27) did not show any kinase activity. Among the phosphorylated proteins there were two phosphorylated molecules whose molecular masses suggested that they could correspond to the previously described PTK, p56<sup>lck</sup>, and p59<sup>fyn</sup>. Generally both PTK become autophosphorylated (32, 40) in in vitro kinase assays, and those labeled bands on the digitonin pattern could correspond to them. To better characterize the tyrosine kinase-associated activity and to confirm the presence of lck and/or fyn PTK, the immunoprecipitates were tested by Western blotting analysis with anti-lck and anti-fyn rabbit antisera. Fig. 7 shows that CD50 mAb coimmunoprecipitated both a 56-kD lck protein and a 59-kD fyn PTK.

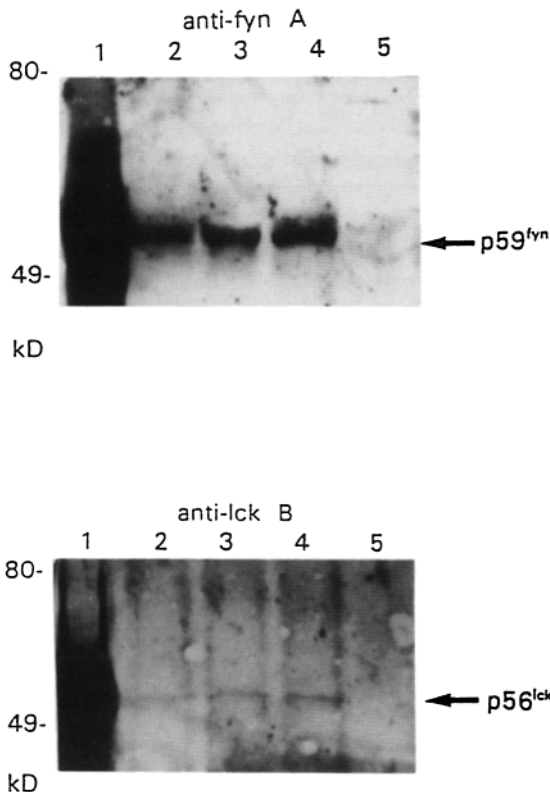
## Discussion

Identified as ICAM-3, an adhesion molecule involved in the initiation of immune responses, the role of CD50 seems restricted to being a new counter-receptor of LFA-1, a main molecule in leukocyte interactions (1, 2). In spite of the resemblances to the other ICAMs, important CD50 differences (cell distribution, cell inductions, intracytoplasmic domain, phosphorylation by PKC stimulation) lead us to suggest a different role for this molecule, probably through its intracytoplasmic domain.

In this paper, data are presented showing that an ICAM molecule, CD50 (ICAM-3), is able to transduce signals in a T cell line. One of the most outstanding characteristics of this signal transduction is the  $\text{Ca}^{2+}$  mobilization. Assays using CD50 and CD3 mAb cross-linking (Fig. 1) allowed



**Figure 6.** Kinase activity associated with immunoprecipitates of CD50. (A) In vitro kinase assay of immunoprecipitates from Triton X-100 lysates: 143-14 (CD27; negative control, lane 1); 101-1D2 (CD50, lane 2); 152-2D11 (CD50, lane 4); and 140-11 (CD50, lane 6). CD50 immunoprecipitates were also analyzed after washing out with 1 M MgCl<sub>2</sub>: 101-1D2 (lane 3); 152-2D11 (lane 5); and 140-11 (lane 7). Arrow shows a 31-kD phosphorylated molecule. (B) Phosphoamino acid analysis of a 31-kD molecule (p31). (C) In vitro kinase assay of CD50 immunoprecipitates from digitonin lysates: 124-1D1 and 143-14 (CD7 and CD27 respectively; negative controls, lanes 1 and 2); 101-1D2 (CD50, lane 3); 152-2D11 (CD50, lane 5); and 140-11 (CD50, lane 7). CD50 immunoprecipitates were also analyzed after washing out with 1 M MgCl<sub>2</sub>: 101-1D2 (lane 4); 152-2D11 (lane 6); and 140-11 (lane 8). Arrows show two phosphorylated molecules, probably corresponding to autophosphorylation of lck and fyn PTK.



**Figure 7.** Western blot analysis of CD50 immunoprecipitates with rabbit polyclonal antiserum to p59<sup>fyn</sup> (A) and p56<sup>lck</sup> (B). Total lysate of Jurkat (lane 1); immunoprecipitates of 101-1D2 (CD50, lane 2), 152-2D11 (CD50, lane 3), 140-11 (CD50, lane 4), and 143-14 (CD27, lane 5, negative control).

us to compare both signaling pathways. The most striking difference is that the CD50 mAb produces a slower, blunter, and steadier increase of Ca<sup>2+</sup> than the CD3 mAb. From the data shown in Fig. 3 B, it comes out that both CD3 and CD50 mAb mobilize Ca<sup>2+</sup> from both sources, that is, internal stores and extracellular input; however, it seems that the extracellular Ca<sup>2+</sup> mobilization, through CD50 mAb stimulation, is more intense and prolonged than the release of intracellular stores. Moreover, the effect of simultaneous stimulation with CD3 and CD50 mAbs (Fig. 3 A) showed an increase of [Ca<sup>2+</sup>]<sub>i</sub> that was more complex than the simple addition of each individual stimulation. These two sets of data, analysis of the Ca<sup>2+</sup> sources and costimulation with CD3 and CD50 mAb, suggest that both stimulating molecules involve both, a common as well as a different signal transduction pathway.

Regarding the factors that regulate Ca<sup>2+</sup> mobilization, from the results presented in this paper, it appears that both IP<sub>3</sub> metabolism and phosphorylation are playing a role. Taking into consideration both, the IP<sub>3</sub> increase and the Ca<sup>2+</sup> mobilization from the internal stores, it appears that the low participation of the intracellular stores could be associated with the low levels of IP<sub>3</sub> observed after CD50 cross-linking. Furthermore, the complete blockade of Ca<sup>2+</sup> uptake in the presence of PTK inhibitors (Herbimycin A and Genistein), suggests that a PTK activity could be associated with CD50. In addition, in vitro kinase assays of CD50 immunoprecipitates showed the presence of a kinase activity associated with CD50 rather than inherent in the CD50 molecule. Moreover, a PTK stimulation was also responsible for

a very important increase of the tyrosine phosphorylation of several substrates including a 33- and a 40-kD proteins. This 40-kD protein could be the equivalent to the substrate observed after CD2 and CD3 stimulation (41). Finally, the Western blot analysis with specific antisera against two well-characterized PTKs, p56<sup>lck</sup>, and p59<sup>fyn</sup>, suggest that both kinases could be involved in CD50 signaling processes. This double association could explain two phosphorylated bands present in the phosphorylation pattern of in vitro kinase assays developed in digitonin detergent conditions. As was previously described, fyn and lck PTKs generally become autophosphorylated upon activation (32, 40). Thus, their presence, on an immunoprecipitation, is detected as phosphorylated molecules when a in vitro kinase assay is performed (Fig. 4 C, arrows). Although a defined membrane molecule has usually been associated exclusively with one of the two PTKs, the simultaneous association of both kinases has also been described with TCR-CD3 (42), CD55, a phosphatidylinositol linked membrane protein (43), and also recently with CD2 (44). These data raise the question about the different roles played by p56<sup>lck</sup> and p59<sup>fyn</sup>. However, taking into account the differential expression of these PTKs on the hematopoietic cells, a different PTK association of CD50 on the different hematopoietic lineages could easily be assumed. In this context, the broad distribution of CD50 molecule could imply a different functional role of CD50 in each kind of cell.

How CD50 interacts with both PTKs is one of the most interesting aspects to be analyzed. In the absence of experimental data, we could assume that the intracytoplasmic domain is responsible for CD50 binding with both PTKs. The interaction between CD50 and the different PTKs could be regulated through CD50 serine phosphorylations, in a similar way as the one that has been described for CD4 molecule. CD4, like CD50, presents three phosphorylated serine residues when T cells are activated by antigen or phorbol esters (45). These serines are placed next to the p56<sup>lck</sup> recognition site in the CD4 molecule (46). However, the characteristic cysteine motif (46) of lck-CD4/CD8 interaction is not present in CD50. Likewise, an indirect association of CD50 with a multimolecular complex formed by the antigen receptor, CD4 or CD8, HLA, CD2, and CD5 (47) could not be ruled out. Moreover, we have observed that when CD50

mAb are co-cross-linked with CD45 mAb, the functional effects of CD50 are abrogated (Fig. 4 C). That means that the function of the CD50-associated PTKs seems to be regulated by CD45 phosphatase.

It is important to point out that the CD50 signal transduction capacity, described in this paper (Ca<sup>2+</sup> mobilization or PTK activity induction), is clearly observed by using both 101-1D2 and 152-2D11 mAbs. These two mAbs recognize two different CD50 epitopes not related to LFA-1 binding (3). In contrast, the mAb 140-11, which recognizes an epitope critical for LFA-1 binding (3), has been shown to be unable to transduce signals in the assays used in this study. In any case, all three CD50 mAbs used were able to coimmunoprecipitate PTK activity as well as p56<sup>lck</sup> and p59<sup>fyn</sup>. Taking into account that certain differences in the affinities of the mAb have been observed (152-2D11 > 101-1D2 > 140-11) (Juan, M., unpublished observation), these differences could be responsible for their different activation capability. However, all these data could also be interpreted by considering that the induction of conformational changes produced by 101-1D2 and 152-2D11 mAbs on CD50 molecule are necessary to induce intracellular signaling. A similar situation has been previously described with other differentiation antigens such as CD2 (48) and CD45 (49), where, depending on the epitope recognized, a different functional effect was observed.

It should also be noted that the transduction of signals could be a bidirectional process, where the binding of CD50 to its counter-receptor, LFA-1, would trigger both molecules to send intracellular signals that would help in the cell activation process. It has also been observed that CD50 cross-linking induced CD25 expression and IL-2 production after PMA stimulation (Juan, M., M. R. Pino-Otín, and E. Martínez-Cáceres, unpublished data). Furthermore, experiments carried out recently in our laboratory, concerning the role of CD50 in lymphocyte-endothelium adhesion and homotypic aggregation (50) showed that CD50 stimulation is able to enhance  $\beta$ 1 and  $\beta$ 2 integrin function in T lymphocytes. These data, together with the data described in this paper, strongly suggest that CD50 (ICAM-3) is not only a counter-receptor for LFA-1, but it is also by itself a functionally relevant signaling molecule.

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