Antibody and B7/BB1-mediated Ligation of the CD28 Receptor Induces Tyrosine Phosphorylation in Human T Cells

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Summary

CD28 is an adhesion receptor expressed as a 44-kD dimer on the surface of a major subset of human T cells. The CD28 receptor regulates the production of multiple lymphokines, including interleukin 2 (IL-2), by activation of a signal transduction pathway that is poorly understood. Here we show that ligation of CD28 by a monoclonal antibody (mAb) or by a natural ligand, B7/BB1, induces protein tyrosine phosphorylation that is distinct from T cell receptor (TCR)-induced tyrosine phosphorylation. CD28-induced protein tyrosine phosphorylation was greatly enhanced in cells that had been preactivated by ligation of the TCR, or by pretreatment with phorbol esters. Rapid and prolonged tyrosine phosphorylation of a single substrate, pp100, was induced in T cells after interaction with B7/BB1 presented on transfected Chinese hamster ovary (CHO) cells. Anti-B7 mAb inhibited B7/BB1 receptor–induced tyrosine phosphorylation, indicating that B7-CD28 interaction was required. CD28-induced tyrosine phosphorylation was independent of the TCR because it occurred in a variant of the Jurkat T cell line that does not express the TCR. Herbimycin A, a protein tyrosine kinase inhibitor, could prevent CD28-induced tyrosine phosphorylation and CD28-induced IL-2 production in normal T cells. The simultaneous crosslinking of CD28 and CD45, a tyrosine phosphatase, could prevent tyrosine phosphorylation of pp100. These results suggest that specific tyrosine phosphorylation, particularly of pp100, occurs directly as a result of CD28 ligand binding and is involved in transducing the signal delivered through CD28 by accessory cells that express the B7/BB1 receptor. Thus, this particular form of signal transduction may be relevant to lymphokine production and, potentially may provide a means to study the induction of self-tolerance, given the putative role of the costimulatory signal in the induction of T cell activation or anergy.

The activation of T lymphocytes requires both an antigen-specific signal that is delivered by the TCR/CD3 complex, and the delivery of accessory cell–derived costimulatory signals (1, 2). While the TCR mediates the specificity of a T cell–initiated immune response, the presence or absence of the costimulatory signal regulates lymphokine gene expression (3, 4). The functional outcome of T cell activation also appears to depend on the nature of the accessory cell–derived costimulatory signal. For example, it has been proposed that specific tolerance can be induced by antigen encountered in the absence of a costimulatory signal (1–3).

CD28, a 44-kD glycoprotein expressed as a homodimer on the surface of most peripheral blood T cells, is a hypothetical receptor for such an accessory signal (5). When CD28+ cells are activated with antigen, mitogenic lectins, anti-TCR/CD3 mAbs, or by PMA, costimulation with CD28 mAbs leads to enhanced production of several lymphokines (IL-2, GM-CSF, IFN-γ, and TNF-α) and increased cellular prolifera-

tion (6, 7). A natural ligand of CD28 has recently been identified as B7/BB1 (8), a cell surface glycoprotein present on activated B cells (9, 10) and IFN-γ-stimulated monocytes (11). Recent experiments in which CD28 was ligated with B7/BB1 indicate potent functional effects of CD28-B7/BB1 interaction that are similar to previous studies that used mAbs to bind the CD28 receptor (12–14).

A major issue that remains unresolved is the nature of the signal transduction pathway utilized by CD28. Triggering of the TCR leads to rapid tyrosine phosphorylation on a variety of substrates, including phospholipase C γ_1, and provides second messengers that activate protein kinase C and raise the intracellular calcium concentration (15). In contrast, CD28 stimulation with bivalent mAb does not appear to be accompanied by increases in [Ca^{2+}]$_i$ and protein kinase C activation (5, 16). Pharmacologic agents also appear to distinguish between signal transduction by the TCR and by CD28, in that TCR-mediated signal transduction is sensitive to the effects of cyclosporine and agents that raise intracellular cAMP concentration, while CD28-mediated effects are resistant (16–18). Furthermore, signal transduction through the TCR appears to require expression of the CD45 tyrosine phosphatase (19) while CD28 can function independently of CD45 expression (20).

It has been demonstrated recently that tyrosine phosphorylation has a pivotal role in the early signal transduction events after engagement of the TCR (21, 22). With regard to the CD28-associated signal transduction pathway, however, the potential involvement of tyrosine phosphorylation has not been extensively studied. The present study reports that stimulation of Jurkat T cells and normal T cells with anti-CD28 mAb induces tyrosine phosphorylation of several substrates. CD28-induced tyrosine phosphorylation was strongly enhanced by prestimulation of cells with anti-CD3 mAb or with phorbol esters. The interaction of cells expressing the B7 molecule with T cells induced prolonged tyrosine phosphorylation of one of these substrates. These effects were specific as mAbs identifying other accessory molecules such as CD5 and MHC class I molecules could not induce tyrosine phosphorylation of this substrate. Finally, the induction of tyrosine phosphorylation by CD28 differed from antigen-induced signal transduction in that it was independent of expression of the TCR.

Materials and Methods

mAbs. Anti-CD3 mAb G19-4 (IgG1), anti-CD28 mAb 9.3 (IgG2a), anti-CD5 mAb 10.2 (IgG2a), and anti-CD45 mAb 9.4 (IgG2a) were produced, purified, and in some cases, biotinylated as described previously (7, 16). Anti-B7 mAb 133 (IgM) was described previously and the dilutions of ascites used are indicated (23). Anti-CD3 mAb OKT3 (IgG2a) was absorbed to goat anti–mouse IgG covalently linked to microspheres (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), by incubation of a 1/10⁵ dilution of pooled ascites with 10⁷ beads/ml in HBSS at room temperature, followed by extensive washing.

Cells. The CD28⁺ subset of T cells was isolated from peripheral blood T lymphocytes by negative selection using immunoabsorption with goat anti–mouse Ig–coated magnetic particles as pre-

viously described (17). This resulted in a population of resting T cells that was >99% CD3⁺ and that did not contain CD2⁺/CD3⁻ cells such as NK cells. The Jurkat T leukemia cell line E6-1 was a gift from Dr. A. Weiss (University of California, San Francisco) and maintained in complete media, i.e., RPMI 1640 containing 2 mM L-glutamine, 50 μg/ml gentamycin, and 10% FCS (HyClone Laboratories, Logan, UT). In some instances, T cells or Jurkat cells were cultured in complete media, or in complete media with 5 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) or OKT3 beads (± 5 beads/cell) before experiments. The Jurkat J32 cell line (CD2⁺,CD3⁺,CD28⁺) has been described (24). J32 variants (CD2⁺,CD3⁻,CD28⁺) were derived by γ irradiation–induced mutagenesis and immunoselection (24); one such cloned mutant, J32-72.4 is stable in culture. The surface receptor expression of these cells was quantitated by indirect immunofluorescence and analyzed by flow cytometry. The mean log fluorescence intensity for each sample was determined and was converted into linear relative fluorescence units (ΔFL) by the formula $\Delta FL = 10^{[(E-C)/D]}$; where E is the mean log fluorescence intensity of the experimental antibody sample, C is the mean log fluorescence intensity of the control antibody sample, and D is 50 channels/decade. For the TCR/CD3 and CD28 receptors, ΔFL of the J32 cells was 27.0 and 57.0, and for the J32-74.2 cells 1.1 and 40.7. Northern blot analysis of J32-72.4 revealed no detectable TCR-β mRNA, while the expression of the TCR-α, CD3-γ, δ, and ε, and TCR ζ mRNA was similar to that of the parental J32 cells (our unpublished data).

B7 Transfection of CHO Cells. CHO cells were transfected with B7 cDNA as previously described (14). These cells have previously been shown to stimulate lymphocyte proliferation and lymphokine secretion in a manner that mimics CD28 mAb–induced T cell activation (13, 14). Transfected CHO cells showing no B7 expression were recloned and are referred to as CHO-B7⁻. CHO cells were detached from tissue culture plates by incubation in PBS with 0.5 mM EDTA for 30 min and fixed in 0.4% paraformaldehyde as previously described (14). Fixed CHO-B7⁻ cells were used as control cells.

Immunoblot Analysis of Protein Tyrosine Phosphorylation. Details of the immunoblot assay with antiphosphotyrosine antibodies have been described elsewhere (25, 26). Cells were suspended at 5–10 × 10⁷ cells/ml in reaction media, i.e., HBSS containing 0.8% FCS and 20 mM Hepes at 37°C at time −3 min and stimulated at time 0 min. mAbs were used at 10 μg/ml final concentration. For cross-linking, biotinylated mAbs were incubated with cells for 5–8 min at room temperature, the cells prewarmed at time −3 min, and stimulated with avidin (Sigma Chemical Co.) at a final concentration of 40 μg/ml at time 0. Stimulation was terminated by the addition of ice-cold 10× lysis buffer, yielding a final concentration of 0.5% Triton X-100 (26). After lysis at 4°C, nuclei were pelleted and postnuclear supernatants were subjected to SDS-PAGE on a 7.5% gel, transferred to polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA), and the membranes probed with affinity-purified antiphosphotyrosine antibodies, labeled with ¹²⁵I staphylococcal protein A (ICN, Irvine, CA) and exposed to x-ray film.

Results

Herbimycin A Prevents CD28-stimulated IL-2 Production. Previous studies have shown that three distinct biochemical signals, provided by phorbol esters, calcium ionophore, and ligation of the CD28 receptor with mAb, are required to cause optimal IL-2 secretion (27). Cells cultured in the pres-
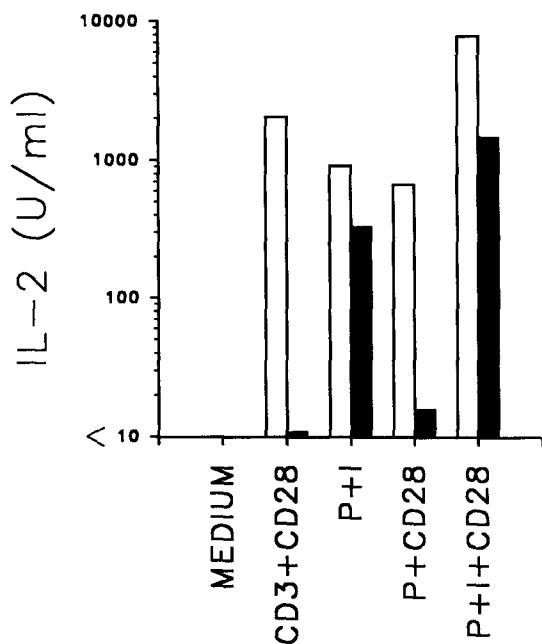
Figure 1. The effect of herbimycin A on CD28-stimulated IL-2 production. T cells were cultured overnight in the absence (*open bars*) or presence (*filled bars*) of herbimycin A (1 μM). The cells were then cultured for a further 24 h in the presence of medium, immobilized anti-CD3 mAb (G19-4), PMA (3 ng/ml) (*P*), or PMA plus ionomycin (150 ng/ml) (*P+I*) in the presence or absence of soluble anti-CD28 mAb 9.3 (1 μg/ml). Cell-free supernatant was collected and serial dilutions were analyzed for IL-2 content by bioassay as described (27). Cells cultured in PMA, ionomycin, or 9.3 mAb only produced <10 U/ml of IL-2.

ence PMA, ionomycin, or CD28 mAb alone produced no detectable IL-2 and, as previously reported (27, 28), stimulation of the CD28 receptor strongly upregulated IL-2 production of T cells stimulated with immobilized anti-CD3 mAb, PMA, or PMA plus ionomycin (Fig. 1). To address the potential role of tyrosine kinases in CD28-triggered signaling, we investigated the effect of herbimycin A, an inhibitor of the *src* family protein tyrosine kinases (29), on the CD28-triggered enhancement of IL-2 production. The CD28 mAb mediated enhancement of IL-2 production in response to stimulation with immobilized anti-CD3, or PMA was nearly completely inhibited in the presence of herbimycin A. In contrast, IL-2 secretion mediated by PMA plus ionomycin stimulation was largely resistant to herbimycin (Fig. 1).

Disruption of the proximal signaling pathway triggered through CD3 could potentially explain the effect of herbimycin on cells stimulated with anti-CD3 and anti-CD28. Consistent with this, CD3-triggered IL-2 production was previously shown to be exquisitely sensitive to herbimycin A (21). However, IL-2 production induced with the combination of PMA plus ionomycin or PMA plus CD28 stimulation permits, in principle, the ability to isolate the CD28 signal for testing the effect of herbimycin A. PMA plus anti-CD28–stimulated IL-2 production was sensitive to the effects of herbimycin A while, as noted above, PMA plus ionomycin–stimulated IL-2 secretion was resistant to the effects of herbimycin A. The
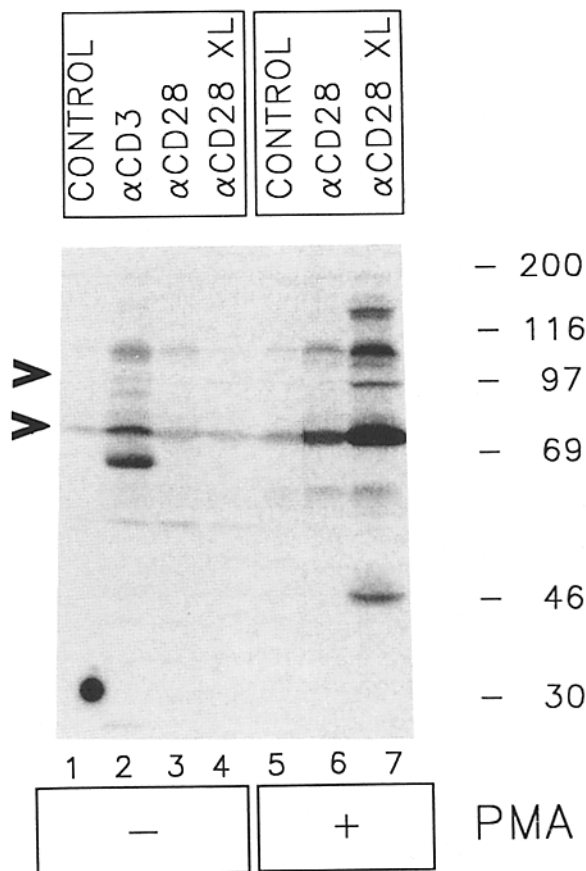
Figure 2. CD28 mAb induces protein tyrosine phosphorylation in PMA-treated Jurkat E-6 cells but not in untreated Jurkat E-6 cells. Jurkat E-6 cells were cultured for 2 d in the presence or absence of PMA (5 ng/ml). After washing, 10⁷ cells in 120 μl were stimulated with reaction media (control), anti-CD3 mAb (G19-4), anti-CD28 mAb (9.3), or crosslinked anti-CD28 mAb (9.3) (final concentration, 10 μg/ml). For crosslinking, biotinylated mAb was added at time −10 min, followed by avidin (40 μg/ml) at time zero. After 2 min, the reaction was terminated with ice-cold lysis buffer and postnuclear supernatants were resolved by SDS-PAGE electrophoresis, transferred to immobilon, and immunoblotted with anti-tiphosphotyrosine, followed by ¹²⁵I–protein A and autoradiography, as described in Materials and Methods. The position of molecular mass markers are shown on the right; arrowheads to the left indicate the position of pp75 and of pp100.

combination of PMA plus ionomycin plus anti-CD28-stimulation resulted in more IL-2 secretion than optimal amounts of PMA plus ionomycin, consistent with previous reports (27, 28). However, in the presence of herbimycin A, PMA plus ionomycin plus CD28–stimulated cells produced approximately equivalent amounts of IL-2 as cells stimulated in the absence of herbimycin with PMA plus ionomycin. Together, the above results suggest that the function of both the TCR and CD28 receptors are sensitive to herbimycin, and further suggest the independent effects of these three reagents on IL-2 gene expression (27, 28).

Crosslinking of the CD28 Receptor by Monoclonal Antibody Induces Protein Tyrosine Phosphorylation in PMA-treated Jurkat and Not in Unstimulated Jurkat Cells. Given the above func-

tional results, the potential involvement of protein tyrosine phosphorylation in CD28-mediated signal transduction was investigated by immunoblot analysis of postnuclear supernatants of whole cell lysates of the T cell leukemia line Jurkat E6-1 (Fig. 2). In a previous report, increased tyrosine phosphorylation could not be detected in resting T cells after crosslinking the CD28 receptor (30). Consistent with that report, no changes in tyrosine phosphorylation were detected in unstimulated Jurkat cells after the binding of bivalent or crosslinked CD28 mAb (Fig. 2, lanes 3 and 4). Previous studies have shown that CD28 stimulation alone does not result in lymphokine production in Jurkat cells or induce proliferation of primary T cells (31–33). Engagement of CD28 by CD28 mAbs or by B7, the natural CD28 ligand, delivers a costimulatory signal provided T cells are stimulated with PMA or with TCR/CD3 mAbs (5, 12–14). We reasoned that CD28-induced protein tyrosine phosphorylation might only occur in the context of a costimulatory signal. To test this hypothesis, Jurkat cells were cultured in PMA and then stimulated with anti-CD28 mAb. In these PMA-stimulated cells, crosslinking of CD28 for 2 min induced phosphotyrosine on substrates migrating with approximate molecular

masses of 47, 62, 75, 82, 100, 110, and 145 kD (Fig. 2, lane 7). Bivalent CD28 mAb induced tyrosine phosphorylation, but to a lesser magnitude. In agreement with a previous report (26), CD3 triggering of Jurkat cells induced tyrosine phosphorylation of pp56, pp65, pp75, pp100, pp110, and pp145 in resting Jurkat cells (Fig. 2, lane 2) and in PMA-treated Jurkat cells (data not shown). Of particular interest are pp75 and pp100, (arrowheads), which are consistently phosphorylated by CD28 stimulation in all conditions we have tested.

CD28 Receptor Crosslinking with Monoclonal Antibody Induces Protein Tyrosine Phosphorylation in Normal T Cells. We performed similar experiments with highly purified peripheral blood T cells from normal human donors in order to determine if CD28 could increase tyrosine phosphorylation in nontransformed cells (Fig. 3). T cells were cultured with PMA for 6 h before CD28 stimulation. Crosslinking of CD28 for 2 min on PMA-treated cells induced the appearance of tyrosine phosphorylated substrates that migrated at 47, 75, and 100 kD (lanes 1 vs. 4). Again, pp75 and pp100 were most prominent and consistently reproduced.

The effects of CD28 stimulation observed after 24–48 h of PMA stimulation (not shown) were more pronounced than those seen after 6 h (Fig. 3). Ligation of CD28 by mAb on resting T cells caused the appearance of weakly detected tyrosine phosphorylation (Fig. 4, *left*). The induction of increased responsiveness to anti-CD28 mAb stimulation by PMA is slow in that 4–6 h of PMA treatment are required to consistently observe CD28-induced tyrosine phosphorylation. Ex-
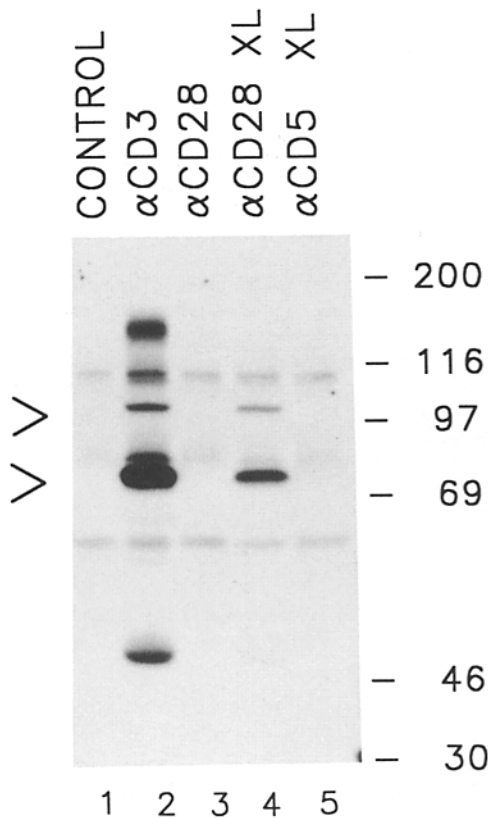
Figure 3. CD28 induces protein tyrosine phosphorylation in PMA-stimulated T cells. Peripheral blood CD28+ T cells were cultured in PMA (5 ng/ml) for 6 h. After washing, 10^7 cells were stimulated for 2 min with media (control), anti-CD3 mAb (G19.4), anti-CD28 mAb (9.3), crosslinked anti-CD28 mAb (9.3), or crosslinked anti-CD5 mAb (10.2). Cells were lysed and protein tyrosine phosphorylation was determined as described in Fig. 2.
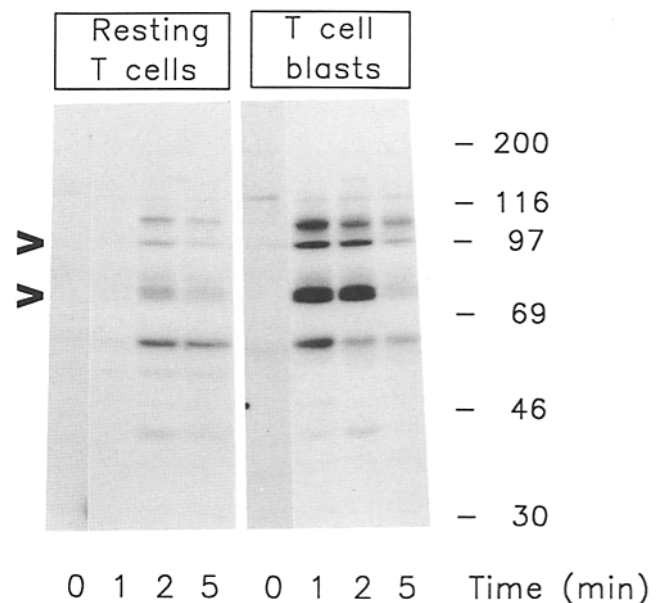
Figure 4. Kinetics of CD28-triggered induction of protein tyrosine phosphorylation in quiescent T cells and in TCR-stimulated T cell blasts. T cells were cultured overnight in medium (*left*) or in the presence of anti-CD3-coated beads (*right*). The cells were recovered, and 8 × 10^6 cells were stimulated with crosslinked anti-CD28 mAb for 0–5 min as indicated, the cells lysed, and protein tyrosine phosphorylation determined as described in Fig. 2.
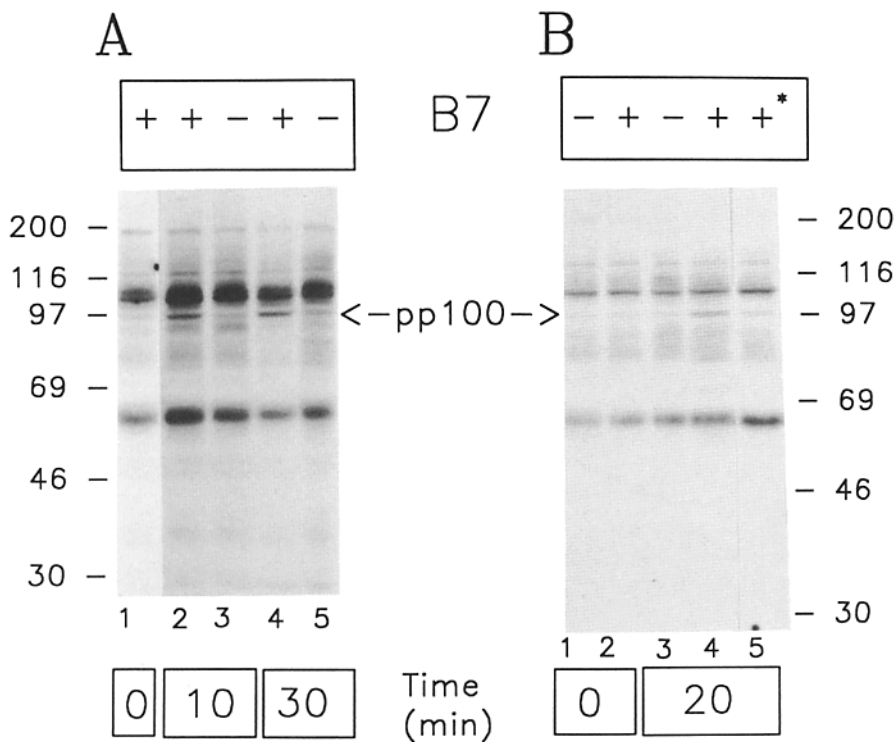
Figure 5. Interaction of CD28 with CHO-B7+ cells induces sustained tyrosine phosphorylation of pp100. CHO cells expressing B7 or not expressing B7 were detached from tissue culture plates and fixed with 0.4% paraformaldehyde as described in Materials and Methods. + and – indicate expression or nonexpression of B7, respectively. T cells were cultured overnight with PMA (5 ng/ml). (A) Time course. 5 × 10⁵ CHO-B7⁻ or B7⁺ cells were added to 5 × 10⁶ PMA-treated T cells at time 0. The reaction was terminated with lysis buffer after 0–30 min. (Lane 1) T cells were lysed first followed by the addition of the CHO-B7⁺ cells. (B) Blocking with anti-B7 mAb. 5 × 10⁵ CHO-B7⁻ cells or B7⁺ cells were added to 5 × 10⁶ PMA-treated T cells at time 0. (Lanes 1 and 2) T cells were lysed first followed by the addition of the CHO-B7⁻ (lane 1) or CHO-B7⁺ (lane 2) cells. (Lane 5) CHO-B7⁺ cells were incubated with anti-B7 mAb 133 (1/100 of ascites) for 30 min before adding them to the T cells (+*). After 20 min, the reaction was terminated and detergent-soluble proteins were processed further as described in Fig. 2. The data in A and B are derived from two independent experiments. The position of pp100 is indicated.

periments with cycloheximide indicate that new protein synthesis is required for cells to become responsive to CD28 (data not shown). The specificity of the CD28-induced tyrosine phosphorylation was investigated by crosslinking CD5 with an isotype-matched mAb (Fig. 3, lane 5). Increased tyrosine phosphorylation on the 75-kD substrate was occasionally induced by CD5 crosslinking. In contrast, CD5 never induced tyrosine phosphorylation on pp100. Similarly, crosslinking of the MHC class I receptor also did not induce tyrosine phosphorylation of this substrate (not shown).

CD28 Receptor Crosslinking Induces Protein Tyrosine Phosphorylation in CD3-treated Normal T Cells. The above experiments suggest that the CD28 receptor is relatively inactive in quiescent cells, and becomes responsive consequent to protein kinase C activation. To determine whether TCR stimulation can also prime cells for the CD28 signal, T cells were cultured overnight in medium or with anti-CD3 mAb OKT3 adsorbed to microspheres (Fig. 4). In this experiment, crosslinked CD28 mAb induced low-level tyrosine phosphorylation on multiple substrates in resting T cells that peaked 2–5 min after CD28 stimulation. In contrast, CD28 mAb induced marked tyrosine phosphorylation in CD3-primed cells that was maximal within 1 min. Thus, costimulation of T cells with anti-CD3 augmented CD28-induced tyrosine phosphorylation as manifested by an increased magnitude of response and an accelerated kinetics of response. This induction of responsiveness to CD28 did not require DNA synthesis, as separate studies have shown that the T cell blasts used for these studies were in the late G₁ phase of the cell cycle (data not shown).

CD28 Receptor–B7/BB1 Receptor Interaction Induces Specific Tyrosine Phosphorylation in T Cells. The above results indicate that CD28 mAb can increase tyrosine phosphorylation on a variety of substrates on pre-activated T cells. Previous studies have indicated that CD28 appears to deliver two biochemically distinct signals, depending on the degree of crosslinking (30). The unique functional properties of CD28 mAb observed after stimulation of T cells do not require highly crosslinked CD28 mAb and are obtained using intact or F(ab')₂ CD28 mAb (33). Recent studies have shown that CHO cells expressing the CD28 ligand mimic the functional effects of CD28 mAb (13, 14). These cells presumably represent a more physiologic means to study CD28 receptor-mediated signal transduction. CHO-B7⁺ cells were incubated with PMA-treated T cells at a CHO/T cell ratio of 1:10 for 5–30 min (Fig. 5 A). B7-transfected CHO cells not expressing B7 on the cell surface (CHO-B7⁻ cells) were used as controls. Before the stimulation, CHO cells were fixed with paraformaldehyde to decrease phosphotyrosine background. Previous studies have indicated that this treatment leaves intact B7-CD28 interaction and the ensuing functional effects (14). For the time zero point, lysis buffer was added to the T cells first, immediately followed by addition of CHO cells to the mixture (Fig. 5 A, lane 1; and B, lanes 1 and 2). CHO-B7⁺ cells induced specific tyrosine phosphorylation that was detected primarily on a substrate that migrated at 100 kD. The CHO-B7-induced tyrosine phosphorylation was detectable within 5 min of stimulation (data not shown) and remained elevated at plateau levels for at least 30 min. CHO-B7-induced tyrosine phosphorylation was evident at
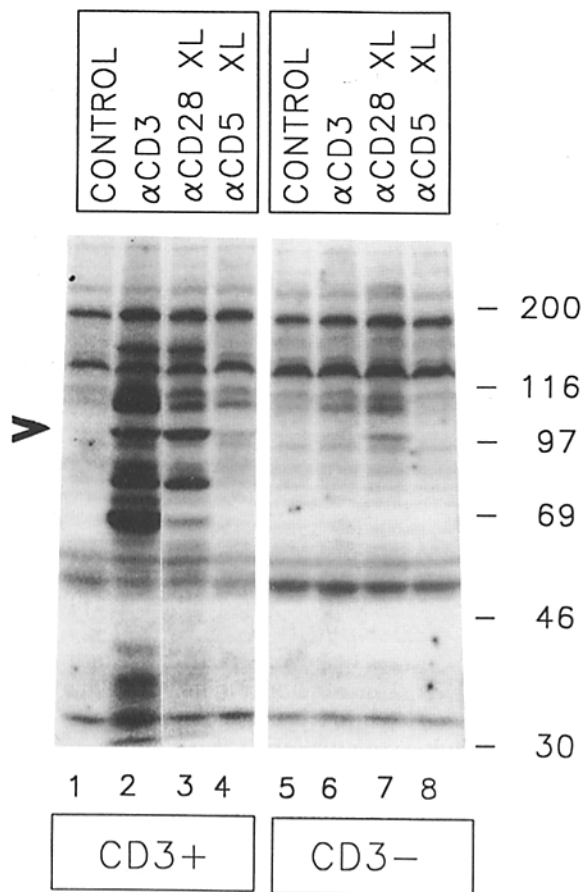
Figure 6. CD28 crosslinking induces tyrosine phosphorylation of pp100 in a CD3⁻ Jurkat cell line. CD3⁺CD28⁺ and CD3⁻CD28⁺ Jurkat mutant cell lines were generated as described in Materials and Methods. 10^7 cells were stimulated for 2 min with anti-CD3 mAb (G19-4), crosslinked CD28 mAb (9.3), or crosslinked anti-CD5 mAb (10.2), as described in Fig. 2. The position of pp100 is indicated.
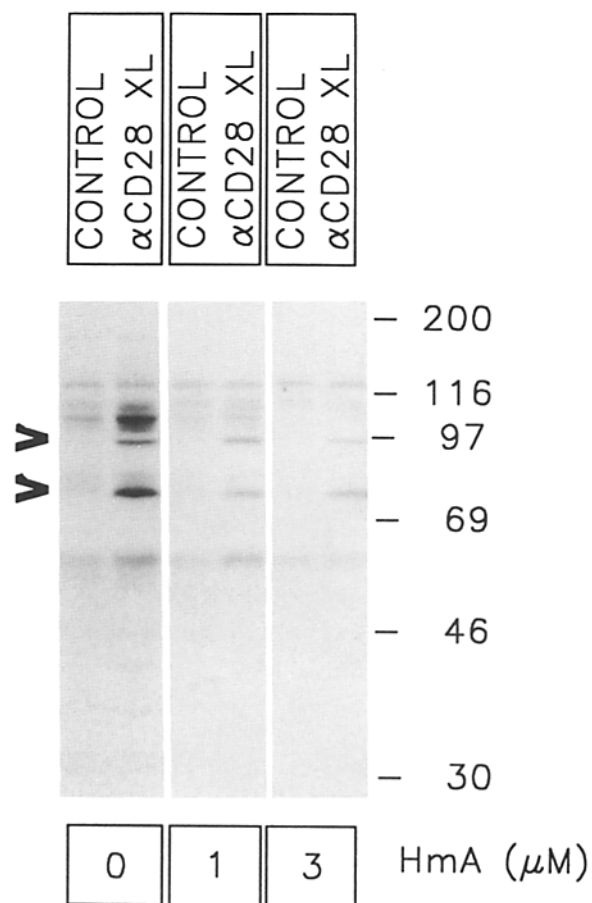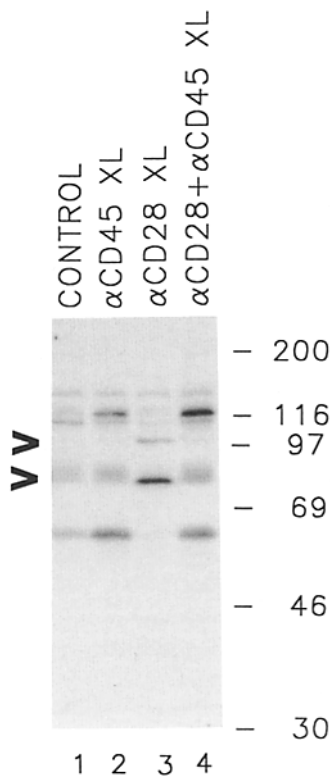

Figure 7. CD28-induced tyrosine phosphorylation can be prevented by herbimycin A. T cells were treated overnight with PMA (5 ng/ml) in the presence of the indicated concentration of herbimycin A or in control medium. The cells were collected, washed, and 8×10^6 cells were stimulated with media or with crosslinked anti-CD28 mAb for 2 min. Detergent-soluble proteins were processed as described in Fig. 2.

a variety of CHO-T cell ratios, and has been consistently observed for only the 100-kD substrate. CHO-B7⁻ cells did not induce tyrosine phosphorylation of pp100. The B7-induced tyrosine phosphorylation was dependent upon CD28-B7 interaction as preincubation of the CHO cells with anti-B7 mAb prevented CHO-B7 induced pp100 tyrosine phosphorylation (Fig. 5 B, lanes 4 vs. 5). B7⁻ CHO cells induced a slight increase in pp100 tyrosine phosphorylation in some experiments, however, this was not consistently observed (Fig. 5, A and B).

In other experiments, alloantigen-induced T cell blasts were tested for CD28-induced tyrosine phosphorylation. T cells were cultured for 8 d with allogeneic irradiated cells and then stimulated with CD28 mAb. Tyrosine phosphorylation that was most pronounced on the 75- and 100-kD substrates was observed (data not shown). Thus, CD28 stimulation of T cells preactivated with alloantigen, CD3 mAb, or PMA can induce tyrosine phosphorylation on a limited number of substrates that are early in onset and brief in duration.

CD28 Receptor–induced Tyrosine Phosphorylation Does Not Require Cell Surface Expression of the T Cell Receptor. There is evidence that the costimulatory signal delivered by CD28 is independent of the TCR. Such conclusions are based on studies of CD28-induced lymphokine production in cell lines lacking expression of the TCR (31). The observation that CD28-induced lymphokine production is resistant to various immunosuppressive agents while TCR-induced lymphokine production is sensitive also suggested that CD28-induced signal transduction might be independent of the TCR (16–18). The Jurkat J32 and J32-72.4 lines were studied to assess whether CD28-induced tyrosine phosphorylation was dependent on expression of the TCR. J32 cells have the wild-type phenotype (24) while J32-72.4 is a TCR⁻ mutant line that expresses 71% of the wild-type levels of the CD28 receptor (see Materials and Methods). CD28 mAb stimulation clearly induced pp100 tyrosine phosphorylation in the TCR⁻ line, although the level of tyrosine phosphorylation was less than in the parental J32 line (Fig. 6). The decreased magnitude

Figure 8. CD28-induced tyrosine phosphorylation can be prevented by anti-CD45 mAb. T cells were cultured overnight with PMA (5 ng/ml). 10^7 cells were incubated for 10 min with media (control), biotinylated anti-CD45 mAb (9.4), anti-CD28 mAb (9.3), or both. mAbs were crosslinked with avidin at time 0. The reaction was terminated after 2 min. Immunoblot analysis with antiphosphotyrosine antibodies of detergent-soluble proteins was performed as described in Fig. 2.

phorylation on pp75 and pp100 that was completely prevented by CD45 (Fig. 8). Consistent with previous results (34), crosslinking of CD45 alone caused increased tyrosine phosphorylation of a 120–135-kD substrate (Fig. 8, lane 2); this effect is also seen in CD28 plus CD45–treated cells. Thus, the above studies indicate that CD28-induced tyrosine phosphorylation is sensitive to an inhibitor of *src* family protein tyrosine kinases, and furthermore, that the CD45 protein tyrosine phosphatase can prevent CD28-induced protein tyrosine phosphorylation.

Discussion

In the present study we have used phosphotyrosine immunoblot analysis to show that ligation of the CD28 receptor induces increased tyrosine phosphorylation on several detergent-soluble cellular substrates. Experiments with an inhibitor of *src* family tyrosine kinases, herbimycin A, suggest that the functional effects of CD28 stimulation on lymphokine gene expression require protein tyrosine phosphorylation. The tyrosine phosphorylation induced by the CD28 receptor differs in several important aspects from that of the TCR. TCR-induced tyrosine phosphorylation occurs in both resting and activated T cells, while CD28-induced tyrosine phosphorylation occurs primarily in previously activated T cells. Ligation of the TCR by antigen or by CD3 mAb induces tyrosine phosphorylation of >12 substrates (26), while CD28 ligation by antibody induces phosphorylation detectable on a more limited set of substrates. Separate experiments failed to demonstrate tyrosine phosphorylation of the TCR ζ chain (not shown). Most striking were the results after CD28 receptor ligation by cell-bound B7, where phosphorylation was consistently detectable on only a single substrate. The specificity of CD28-induced tyrosine phosphorylation was shown by the failure of isotype-matched mAbs to two additional T cell adhesion receptors, CD5 (Fig. 3) and MHC class I antigens (not shown), to induce tyrosine phosphorylation on pp100. Thus, there are several cellular adhesion receptors that have "accessory" functions on T cells, and yet do not activate the same biochemical signal transduction cascade associated with the CD28 receptor.

Experiments using the Jurkat E6-1 T cell line indicated there is an absolute requirement for PMA pretreatment in order to observe CD28-induced tyrosine phosphorylation. In contrast, there was no requirement for cellular preactivation in the Jurkat J32 line, while, as noted above, there is a relative requirement for PMA or TCR prestimulation of normal T cells in order to induce CD28 responsiveness. The basis for the prestimulation requirement is not known at this time, although we have noted that new protein synthesis is required. Previous studies have shown that antigen treatment of T cell clones causes substantial enhancement of CD28 receptor expression (35), and in the case of primary T cells, PMA treatment causes increased CD28 mRNA expression and increased surface CD28 expression (36). However, increased surface CD28 expression is unlikely to explain the requirement for new protein synthesis for CD28-induced signal

of CD28-induced phosphorylation in the TCR⁻ line is most likely the result of decreased surface expression of CD28 in these cells. TCR stimulation caused substantial stimulation of a variety of phosphoproteins in J32 cells, while, as expected, anti-CD3 stimulation of the TCR⁻ J32-72.4 line failed to induce significant tyrosine phosphorylation. In J32 cells the magnitude of the phosphorylation observed on pp100 was nearly equivalent after TCR and CD28 stimulation. The substrates induced by CD28 stimulation all comigrated with CD3-induced substrates. However, there are many phosphorylated substrates after CD3 stimulation that are not detected after CD28 stimulation. CD5 stimulation failed to cause pp100 tyrosine phosphorylation in either line.

CD28-induced Tyrosine Phosphorylation Can Be Prevented by CD45 and by Herbimycin A. Given that protein tyrosine kinase inhibitor herbimycin A could efficiently inhibit CD28-induced IL-2 secretion (Fig. 1), we tested this inhibitor for effects on CD28-induced tyrosine phosphorylation. T cells were cultured in PMA and various concentrations of herbimycin A. Tyrosine phosphorylation induced by anti-CD28 mAb was nearly completely prevented in herbimycin-treated cells (Fig. 7) under conditions that specifically inhibit CD28-induced IL-2 production (Fig. 1).

The brief temporal course of CD28 mAb–induced tyrosine phosphorylation (Fig. 4) suggests regulation by a phosphatase. To address the effects of phosphatases on CD28-mediated signal transduction, T cells were cultured in PMA and then stimulated with crosslinked CD28, CD45, or CD28 plus CD45 mAbs. CD28 crosslinking induced tyrosine phos-

transduction, as Jurkat cells have brighter CD28 surface expression than primary T cells, and yet have no detectable tyrosine phosphorylation after CD28 stimulation. Thus, the coupling of the CD28 receptor to signal transduction likely involves the expression of some other protein in the signal transduction pathway that is limiting or absent in resting T cells, and is induced by TCR stimulation.

The requirement for PMA or anti-CD3 pretreatment could indicate either that CD28-associated signal transduction requires a consequence of PKC activation, or alternatively, that depletion of PKC is required for the CD28 signal. The latter possibility is considered unlikely as only relatively low concentrations of PMA are required, and furthermore, a more physiologic means of activation, anti-CD3, is capable of inducing CD28 responsiveness. In addition, immunoblot analysis of CD28-responsive cells indicates easily detectable amounts of PKC.

Studies with Jurkat mutants further indicate that CD28-induced tyrosine phosphorylation (Fig. 6) and biologic function (31) can occur in the absence of the TCR. In this respect, CD28 appears to be unique, in that other accessory molecules involved in T cell activation such as CD2, Ly-6, Thy-1, and CD5 appear to require the presence of the TCR. The mechanism for the independence of the CD28 pathway remains unclear, and may indicate that the CD28 receptor is coupled to different kinase(s)/phosphatases than is the TCR. This notion would be in accord with findings of Koretzky et al. (20), who found that IL-2 production induced by anti-CD28 mAb was independent of CD45 expression, while both CD2- and TCR-induced IL-2 expression required CD45 expression. The independence of the CD28-induced tyrosine phosphorylation from the TCR is also in accord with studies that indicated sensitivity of TCR-induced functional responses to cyclosporine, prostaglandin E_2, 1,25-hydroxyvitamin D_3, and cholera toxin, and resistance of the CD28 pathway to these agents (16–18, 37).

In previous studies, we and others have failed to observe tyrosine phosphorylation after CD28 receptor stimulation (30). There appear to be several reasons for the differing results, including the failure to test appropriately "primed" T cells and the fact that CD28 stimulation causes only a minor subset of substrate phosphorylation. The inability in the present studies to consistently observe CD28 mAb–induced tyrosine phosphorylation with bivalent antibody is likely explained by the signal being at the limit of detection by our current assay system. However, the observation that cell-bound B7/BB1 receptor could activate T cell tyrosine phosphorylation suggests that tyrosine phosphorylation results from CD28 receptor–ligand interaction under physiologic conditions.

The CD45 tyrosine phosphatase has previously been shown to prevent calcium mobilization induced by crosslinked CD28 (38), and in the current studies, we have found that under these conditions, CD28-induced tyrosine phosphorylation of pp100 is prevented. This result may imply that CD28-induced tyrosine phosphorylation can be regulated by a tyrosine phosphatase. Under physiologic conditions, it is likely that phosphatases other than CD45 regulate CD28-mediated signal transduction, as CD28-induced lymphokine production has been shown to occur in CD45-negative Jurkat cells (20).

The CD28 receptor has been shown to have potent effects on lymphokine production by several different mechanisms (28, 39), and postulated to have effects on thymic differentiation (40) and T cell anergy (3, 41). The relation of the CD28-induced tyrosine phosphorylation to these functional effects remains to be demonstrated in further studies.

References

1. Lafferty, K.J., L. Andrus, and S.J. Prowse. 1980. Role of lymphokine and antigen in the control of specific T cell responses. Immunol. Rev. 51:279.
2. Steinman, R.M., and J.W. Young. 1991. Signals arising from antigen-presenting cells. Curr. Opin. Immunol. 3:361.
3. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell an-

tigen receptor occupancy. *Annu. Rev. Immunol.* 7:445.

4. Kohno, K., Y. Shibata, Y. Matsuo, and J. Minowada. 1990. CD28 molecule as a receptor-like function for accessory signals in cell-mediated augmentation of IL-2 production. *Cell. Immunol.* 131:1.

5. June, C.H., J.A. Ledbetter, P.S. Linsley, and C.B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol. Today.* 11:211.

6. Thompson, C.B., T. Lindsten, J.A. Ledbetter, S.L. Kunkel, H.A. Young, S.G. Emerson, J.M. Leiden, and C.H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA.* 86:1333.

7. Ledbetter, J.A., P.J. Martin, C.E. Spooner, D. Wofsy, T.T. Tsu, P.G. Beatty, and P. Gladstone. 1985. Antibodies to Tp67 and Tp44 augment and sustain proliferative responses of activated T cells. *J. Immunol.* 135:2331.

8. Linsley, P.S., E.A. Clark, and J.A. Ledbetter. 1990. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proc. Natl. Acad. Sci. USA.* 87:5031.

9. Yockochi, T., R.D. Holly, and E.A. Clark. 1982. B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. *J. Immunol.* 128:823.

10. Freeman, G.J., A.S. Freedman, J.M. Segil, G. Lee, J.F. Whitman, and L.M. Nadler. 1989. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* 143:2714.

11. Freedman, A.S., G.J. Freeman, K. Rhynhart, and L.M. Nadler. 1991. Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. *Cell. Immunol.* 137:429.

12. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. *J. Exp. Med.* 173:759.

13. Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.

14. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sugita, A.S. Freedman, C. Morimoto, and L.M. Nadler. 1991. B7 provides a costimulatory signal through its ligand CD28 which induces simulated T cells to proliferate and secrete interleukin-2. *Proc. Natl. Acad. Sci. USA.* 88:6575.

15. Klausner, R.D., and L.E. Samelson. 1991. T cell antigen receptor activation pathways, the tyrosine kinase connection. *Cell.* 64:875.

16. Ledbetter, J.A., M. Parsons, P.J. Martin, J.A. Hansen, P.S. Rabinovitch, and C.H. June. 1986. Antibody binding to CD5 (Tp67) and Tp44 T cell surface molecules: effects on cyclic nucleotides, cytoplasmic free calcium and cAMP mediated suppression. *J. Immunol.* 137:3299.

17. June, C.H., J.A. Ledbetter, M.M. Gillespie, T. Lindsten, and C.B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7:4472.

18. Bjorndahl, J.M., S.S. Sung, J.A. Hansen, and S.M. Fu. 1989. Human T cell activation: differential response to anti-CD28 is compared to anti-CD3 monoclonal antibodies. *Eur. J. Immunol.* 19:881.

19. Koretzky, G.A., J. Picus, M.L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell an-

tigen receptor to the phosphatidyl inositol pathway. *Nature (Lond.).* 346:66.

20. Koretzky, G.A., J. Picus, T. Schultz, and A. Weiss. 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. USA.* 88:2037.

21. June, C.H., M.C. Fletcher, J.A. Ledbetter, G.L. Schieven, J.N. Siegel, A.F. Phillips, and L.E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA.* 87:7722.

22. Mustelin, T., K.M. Coggeshall, N. Isakov, and A. Altman. 1990. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science (Wash. DC).* 247:1584.

23. Freedman, A.S., G. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. B7, a B cell-restricted antigen that identifies preactivated B cells. *J. Immunol.* 139:3260.

24. Makni, H., J.S. Malter, J.C. Reed, S. Nobuhiko, G. Lang, D. Kioussis, G. Trinchieri, and M. Kamoun. 1991. Reconstitution of an active surface CD2 by DNA transfer in CD2-CD3+ Jurkat cells facilitates CD3-T cell receptor-mediated IL-2 production. *J. Immunol.* 146:2522.

25. Hsi, E.D., J.N. Siegel, Y. Minami, E.T. Luong, R.D. Klausner, and L.E. Samelson. 1989. T cell activation induces rapid tyrosine phosphorylation of a limited number of cellular substrates. *J. Biol. Chem.* 264:10836.

26. June, C.H., M.C. Fletcher, J.A. Ledbetter, and L.E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144:1591.

27. June, C.H., J.A. Ledbetter, T. Lindsten, and C.B. Thompson. 1989. Evidence for the involvement of three distinct signals in the induction of IL-2 gene expression in human T lymphocytes. *J. Immunol.* 143:153.

28. Fraser, J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science (Wash. DC).* 251:313.

29. Uehara, Y., H. Fukazawa, Y. Murakami, and S. Mizuno. 1989. Irreversible inhibition of V-src tyrosine kinase activity by Herbimycin A and its abrogation by sulfhydryl compounds. *Biochem. Biophys. Res. Commun.* 163:803.

30. Ledbetter, J.A., J.B. Imboden, G.L. Schieven, L.S. Grosmaire, P.S. Rabinovitch, T. Lindsten, C.B. Thompson, and C.H. June. 1990. CD28 ligation in T-cell activation, evidence for two signal transduction pathways. *Blood.* 75:1531.

31. Weiss, A., B. Manger, and J. Imboden. 1986. Synergy between the T3/antigen receptor complex and Tp44 in the activation of human T cells. *J. Immunol.* 137:819.

32. Martin, P.J., J.A. Ledbetter, Y. Morishita, C.H. June, P.G. Beatty, and J.A. Hansen. 1986. *J. Immunol.* 136:3282.

33. Hara, T., S.M. Fu, and J.A. Hansen. 1985. Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44-kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* 161:1513.

34. Samelson, L.E., M.C. Fletcher, J.A. Ledbetter, and C.H. June. 1990. Activation of tyrosine phosphorylation in human T cells via the CD2 pathway. Regulation by the CD45 tyrosine phosphatase. *J. Immunol.* 145:2448.

35. Lesslauer, W., F. Koning, T. Ottenoff, M. Gipmart, E. Goulmy, and J.J. van Rood. 1986. T90/44 (9.3 antigen). A cell surface molecule with a function in human T cell activation. *Eur. J. Immunol.* 16:1289.

36. Turka, L.A., J.A. Ledbetter, K. Lee, C.H. June, and C.B.

Thompson. 1990. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. *J. Immunol.* 144:1646.

37. Vanham, G., J.L. Ceuppens, and R. Bouillon. 1989. T lymphocytes and their CD4 subset are direct targets for the inhibitory effect of calcitriol. *Cell. Immunol.* 124:320.

38. Ledbetter, J.A., N.K. Tonks, E.H. Fischer, and E.A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. *Proc. Natl. Acad. Sci. USA.* 85:8628.

39. Lindsten, T., C.H. June, J.A. Ledbetter, G. Stella, and C.B.

Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science (Wash. DC).* 244:339.

40. Turka, L.A., J.A. Ledbetter, K. Lee, C.H. June, and C.B. Thompson. 1990. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. *J. Immunol.* 144:1646.

41. O'Hehir, R.E., and J.R. Lamb. 1990. Induction of specific clonal anergy in human T lymphocytes by *Staphylococcus aureus* enterotoxins. *Proc. Natl. Acad. Sci. USA.* 87:8884.