Differential Association of Protein Tyrosine Kinases with the T Cell Receptor Is Linked to the Induction of Anergy and Its Prevention by B7 Family-mediated Costimulation

By Vassiliki A. Boussiotis, Dwayne L. Barber,* Bohyung J. Lee, John G. Gribben, Gordon J. Freeman, and Lee M. Nadler

*From the Division of Hematologic Malignancies and *Pediatric Oncology, Dana-Farber Cancer Institute and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115*

Summary

When stimulated through their antigen receptor, without costimulation, T cells enter a state of antigen-specific unresponsiveness, termed anergy. B7-mediated costimulation, signaling via CD28, is sufficient to prevent the induction of anergy. Here we show that ligation of T cell receptor (TCR) by alloantigen alone, which results in anergy, activates tyrosine phosphorylation of TCRζ and its association with fyn. In contrast, TCR ligation in the presence of B7 costimulation, which results in productive immunity, activates tyrosine phosphorylation of TCRζ and CD3 chains, which associate with activated lck and zeta-associated protein (ZAP) 70. Under these conditions, CD28 associates with activated lck and TCRζ. These data suggest that the induction of anergy is an active signaling process characterized by the association of TCRζ and fyn. In addition, CD28-mediated costimulation may prevent the induction of anergy by facilitating the effective association of TCRζ and CD3ε with the critical protein tyrosine kinase lck, and the subsequent recruitment of ZAP-70. Strategies to inhibit or activate TCR-associated, specific protein tyrosine kinase–mediated pathways may provide a basis for drug development with potential applications in the fields of transplantation, autoimmunity, and tumor immunity.

To induce a productive T cell immune response, signaling through the TCR complex must result in T cell activation, cytokine secretion, and clonal expansion. These events are mediated by a complex biochemical cascade initiated by phosphorylation of the TCR complex subunits, activation of protein tyrosine kinases (PTKs)[1], protein phosphorylation, and increased intracellular Ca^{2+} levels (1). Although it is not clear which PTKs are involved in the initial signaling, there is compelling evidence that the tyrosine kinases fyn, lck, and zeta-associated protein (ZAP) 70 are implicated in the early signaling events (2–9). High efficiency TCR cross-linking by mAbs or high density antigen can induce the above mentioned biochemical events and generate a productive T cell response in vitro.

However, under physiologic conditions, suboptimal cross-linking of TCR by antigen alone is frequently not sufficient to induce a productive immune response, but instead leads to long-term, antigen-specific unresponsiveness, termed anergy (10). Under these conditions, T cells require a contact-dependent costimulatory signal provided by the APCs (11–13). Increasing evidence demonstrates that one such critical costimulatory signal is provided by members of the B7 family to their ligand, CD28, on the T cells (14–17). B7-mediated costimulation is sufficient to prevent the induction of anergy (18–20). In the absence of B7 costimulation, cytokines (20–22) can also prevent the induction of T cell anergy. It appears that their role is mediated via the common γ chain of the IL-2, IL-4, IL-7, and IL-15 receptors (23). Although the functional role of B7 costimulation is well established, the proximal biochemical events associated with the induction of anergy and its prevention by B7 costimulation are ill understood.

We attempted to determine the biochemical events associated with the induction of anergy by alloantigen and induction of productive immunity by alloantigen in the presence of B7-mediated costimulation. We examined activation of protein tyrosine phosphorylation, the phosphorylation status of the TCR components, and the tyrosine kinase activity associated with TCRζ, CD3ε, and CD28. In the results to be reported below, we demonstrate that during the induction of anergy, although protein tyrosine phosphorylation is activated, CD3ε is not phosphorylated whereas TCRζ is only partially phosphorylated and associates with fyn. In contrast, during the induction of productive immu-

[1]*Abbreviations used in this paper:* PTK, protein tyrosine kinase; ZAP-70, zeta-associated protein 70.

nity, there is activation of tyrosine phosphorylation, phosphorylation of ϵ and hyperphosphorylation of ζ chains of the TCR, both of which associate with lck and ZAP-70. Moreover, simultaneous TCR and CD28 ligation by antigen and B7, respectively, results in association of CD28 with TCRζ and lck. Under these conditions, lck appears to be involved in the phosphorylation of TCRζ, potentially providing the critical tyrosine kinase activity necessary for T cells to proceed to successful TCR signaling.

Materials and Methods

Human T Cell Clones. HLA-DR7 alloantigen-specific T cell clones TC-1, TC-2, and TC-3 ($CD4^+$, $CD8^-$, $CD28^+$, $B7^-$) were generated as previously described (24). T cell clones were rested for 10–14 d in IL-2 without alloantigen restimulation and before use, cells were cultured overnight in media. LBL-DR7 is an EBV-transformed lymphoblastoid B cell line homozygous for HLA-DR7 and expresses adhesion and costimulatory molecules including B7-1 (CD80), B7-2 (CD86), LFA-1 (CD11a), LFA-3 (CD58), and intercellular adhesion molecule 1 (CD54). All experiments described here were done with three clones.

NIH 3T3 Transfected Cells. Alloantigen and/or costimulatory molecules were presented by use of NIH 3T3 stable transfectants (t-) that were constructed as previously described (19, 25). Transfectants expressing similar levels of DR and/or B7-1 or B7-2 were selected for use.

Antibodies and Fusion Proteins. The rabbit antipeptide antiserum (N-23), used to immunoprecipitate TCRζ chain, was kindly provided by Dr. E. Reinherz (Harvard Medical School); anti–human TCRζ mAb (TIA-2) (26), used to detect nonphosphorylated TCRζ by immunoblot, was kindly provided by Dr. P. Anderson (Dana-Farber Cancer Institute, Harvard Medical School); anti–human CD3ϵ mAb (OKT3) that binds to human CD3ϵ subunit (27), was purchased from American Type Culture Collection (Rockville, MD); anti-CD28 (9.3) mAb was kindly provided by Dr. C. June (Naval Research Institute, Bethesda, MD); antiphosphotyrosine mAb (4G10) (Upstate Biotechnology, Inc., Lake Placid, NY) was used for immunoblotting at 1:2,000; anti-lck and anti-fyn antisera (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoprecipitations at 2 μl/test and for immunoblotting at 1:1,000. CTLA4-Ig is a fusion protein consisting of the extracellular domain of CTLA4 fused to the CH2 and CH3 domains of human IgG, and is a potent inhibitor of B7-1– and B7-2–mediated costimulation. CTLA4-Ig was constructed and used as previously described (19).

Primary and Secondary Stimulation. T cell clones were incubated in 24 well plates (Nunc, Roskilde, Denmark) in a primary culture with either t-DR7, t-DR7/B7-1, t-DR7/B7-2, LBL-DR7, or LBL-DR7+CTLA4-Ig. After 48 h of primary culture, T cell clones were separated from LBL-DR7 by Ficoll density centrifugation and from transfectants by Percoll gradient centrifugation, and cultured in media without IL-2 for 24 h. Each population was subsequently rechallenged with LBL-DR7 at a 1:1 stimulator/responder ratio. Before use, LBL-DR7 and transfectants were treated with mitomycin-C as previously described (23).

Thymidine Incorporation. During the last 16 h of a 72-h culture period, cells were incubated with 1 μCi (37 kBq) of [*methyl*-^{3}H] thymidine (Du Pont, Boston, MA). Thymidine incorporation was assessed as previously described (23) and was used as an index of mitogenic activity.

Tyrosine Phosphorylation and Immunoprecipitations. For activation of tyrosine phosphorylation, transfected stimulators were treated with mitomycin-C and cultured overnight in 24-well plates. T cell clones were added to the adhered transfectants at a ratio of 1:1, precipitated by fast spin, and cultured at 37°C. Kinetics experiments for various time intervals (30 s–30 min) showed that activation of tyrosine phosphorylation was first observed at 1 min, peaked at 10 min, and declined thereafter (unpublished results). Therefore, in all subsequent experiments, activation of protein tyrosine phosphorylation in whole cell lysates was examined at 10 min of culture. Stimulation was stopped with cold wash buffer containing PBS, 5 mM EDTA, 10 mM NaF, and 0.4 mM Na_3VO_4. Cells were transferred into Eppendorf tubes and lysed with lysis buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, aprotinin (5 μg/ml), pepstatin (1 μg/ml), soybean trypsin inhibitor (2 μg/ml), 1 mM PMSF, 0.5% Brij 96, and 0.5% NP-40 (Sigma Chemical Co., St. Louis, MO). Whole cell lysates (2×10^6 T cell equivalents/lane) were analyzed on 12.5% gels by SDS-PAGE. Immunoprecipitations (10^7 cell equivalents/condition) were performed with anti-TCRζ (N-23) antiserum (1 μl/sample) or anti-CD3ϵ mAb (2 μg/sample); proteins were analyzed by 10% SDS-PAGE; and immunoblotting, stripping, and reblotting of the immunoblot were done as previously described (23). Immunoblotting was performed with: antiphosphotyrosine mAb (1:2,000); anti-TCRζ mAb (TIA-2, 1:2,000); specific antisera for ZAP-70, lck, and fyn (1:2,000) (Santa Cruz Biotechnology), followed by horseradish peroxidase anti–mouse IgG (1:3,000) for anti-phosphotyrosine and anti-TCRζ immunoblots, horseradish peroxidase–protein A (1:5,000, Amersham Corp., Arlington Heights, IL) for lck and fyn, or by horseradish peroxidase anti–rabbit IgG (1:10,000, Promega Corp., Madison, WI) for ZAP-70 immunoblots. Immunodetection was subsequently performed by enhanced chemiluminescence (Amersham Corp.). For two-dimensional electrophoresis, after stimulation, samples were lysed in either digitonin (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, aprotinin [5 μg/ml], pepstatin [1 μg/ml], soybean trypsin inhibitor [2 μg/ml] 1 mM PMSF, and 1% digitonin) or NP-40 lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, aprotinin [5 μg/ml], pepstatin [1 μg/ml], soybean trypsin inhibitor [2 μg/ml] 1 mM PMSF, and 1% NP-40), immunoprecipitated with anti-CD3ϵ or anti-ζ antibodies, and subjected to two-dimensional (nonreducing/reducing) SDS-PAGE as described (26). Proteins were transferred on nitrocellulose membranes and subjected to antiphosphotyrosine immunoblot.

Cell Surface Radio Iodination and Immunoprecipitation. Cells were washed, resuspended in 200 μl cold PBS, and labeled with ^{125}I (1 mCi per sample) by the lactoperoxidase method (28). After washing three times in PBS to remove free iodine, cells were solubilized in digitonin lysis buffer precleared overnight with protein A–Sepharose (50 μl/test; Pharmacia Biotech, Uppsala, Sweden), 10% (vol/vol), and rabbit anti–mouse Ig. Immunoprecipitations were carried out in 500 μl lysis buffer using anti-CD3 mAb and rabbit anti–mouse Ig (1 μg/mg) for 2 h at 4°C; immune complexes were isolated on protein A–Sepharose (50 μl/test), and analyzed by two-dimensional SDS-PAGE. Gels were dried and radiolabeled proteins were examined by autoradiography.

In Vitro Kinase Reactions and Phosphoamino Acid Analysis. For in vitro kinase assays cells were stimulated for 5 min at 37°C and lysates were prepared as above. Kinetics experiments for various time intervals (30 s–30 min) in our system showed that tyrosine kinase activation was first observed at 30 s, peaked at 5 min, and

declined thereafter (unpublished results). Therefore, in all subsequent experiments, PTK activation was examined at 5 min of culture. Cells were cultured as indicated, lysates were prepared, immunoprecipitations (5×10^6/condition) were conducted with anti-TCRζ antiserum, anti-CD3ε, or anti-CD28 (2 μg/sample) mAb, immune complexes were isolated on protein A–Sepharose (50 μl/sample), and in vitro kinase reactions were performed as previously described (29). Briefly, samples were washed twice in lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, aprotinin [5 μg/ml], pepstatin [1 μg/ml], soybean trypsin inhibitor [2 μg/ml] 1 mM PMSF, 0.5% Brij 96, and 0.5% NP-40) and once in kinase buffer (20 mM Hepes, pH 7.2, 5 mM $MgCl_2$, and 5 mM $MnCl_2$). The immunprecipitates were then suspended in 40 μl kinase buffer containing 10 μCi of γ-[^{32}P]ATP and incubated at room temperature for 15 min. Reactions were terminated by the addition of 10 mM Hepes, 10 mM EDTA, washed twice with double distilled H_2O, analyzed by 12.5% SDS-PAGE, and transferred to polyvinyl difluoride (PVDF) membranes. When indicated, membranes were incubated with 1 M KOH for 1 h at 50°C, and exposed to x-ray films. For analysis of reprecipitated antigen, the immune kinase reaction mixture was supplemented with 1% SDS, boiled for 5 min and diluted to 0.1% SDS with lysis buffer as described previously (30). The denatured immunoprecipitates were reprecipitated with the indicated antibodies.

Phosphoaminoacid analysis was performed as previously described (31). Briefly, in vitro phosphorylated proteins identified by autoradiography were excised from the PVDF membrane, isolated by acid hydrolysis, and analyzed by one-dimensional TLC. The identity of the radioactive species was determined by comparison to the position of phosphoaminoacid standards that were visualized by ninhydrin.

Results

Differential Patterns of Tyrosine Phosphorylation are Generated after Signals that Are Associated with the Induction of Productive Immunity or Anergy. Alloantigen-specific T cell clones were primarily cultured with either t-DR7, t-DR7/B7-1, t-DR7/B7-2, or LBL-DR7 in the absence or the presence of CTLA4-Ig. To determine whether the various primary culture conditions induced anergy or productive immunity, T cell clones were rechallenged with LBL-DR7 stimulators. Primary culture in the presence of B7-mediated costimulation (t-DR7/B7-1, t-DR7/B7-2, or LBL-DR7) resulted in a significant secondary response on rechallenge with LBL-DR7. In contrast, presentation of alloantigen in the primary culture in the absence of B7-mediated costimulation (t-DR7 or LBL-DR7+CTLA4-Ig) resulted in anergy (Fig. 1 *A*).

Because activation of protein tyrosine phosphorylation is the earliest biochemical event occurring after T cell stimulation (32), we examined whether protein tyrosine phosphorylation was activated after immunogenic or anergizing stimulation by alloantigen. T cell clones were cultured with the same stimulators and protein tyrosine phosphorylation was examined in whole cell lysates by antiphosphotyrosine immunoblot. Activation of protein tyrosine phosphorylation was observed under all culture conditions, but distinct patterns of protein tyrosine phosphorylation were activated by anergizing or immunogenic stimulation (Fig. 1 *B*). These results demonstrated that both induction of anergy and productive immunity are associated with activation of signaling events.

Multiple Chains of TCR Become Tyrosine Phosphorylated after Stimulation by Alloantigen and B7 Costimulation, Whereas only TCRζ Chain Becomes Phosphorylated after Stimulation by Alloantigen Alone. After T cell activation γ, δ, ε, and ζ chains of the TCR become phosphorylated (33–37). In light of the differential patterns of protein tyrosine phosphorylation noted in Fig. 1 *B* and the potential that some of these might represent components of the TCR complex, we examined whether TCR components were tyrosine phosphorylated after stimulation by HLA-DR7 alloantigen with or without B7 mediated costimulation. Fig. 1 *C* depicts the TCR components of the TC-1 clone as determined by surface ^{125}I labeling, anti-CD3ε immunoprecipitation, and two-dimensional gel electrophoresis, under conditions that do not dissociate the TCR components. To determine which TCR components are tyrosine phosphorylated after stimulation by alloantigen with or without B7 mediated costimulation, T cells were cultured with either t-DR7, t-DR7/B7-1, or t-DR7/B7-2; lysates were prepared in digitonin lysis buffer, immunoprecipitated with anti-CD3ε mAb and analyzed by two-dimensional nonreducing/reducing gel electrophoresis and antiphosphotyrosine immunoblot. In comparison with clones cultured in media (Fig. 1 *D*), t-DR7 resulted in significant phosphorylation of the TCRζ chain and the generation of a major 21-kD and a barely discernible 18-kD phosphoprotein (Fig. 1 *E*). In contrast, simultaneous stimulation via TCR and B7 (t-DR7/B7-1, Fig. 1 *F* or t-DR7/B7-2, data not shown) resulted in phosphorylation of not only TCRζ but also of TCR γ, δ, and ε. Moreover, under these culture conditions, the pattern of electrophoretic migration of the phosphorylated TCRζ chain was distinct from that induced by t-DR7 and resulted in the generation of multiple phosphoproteins with electrophoretic migration ranging between 16 and 22 kD (Fig. 1 *F*). Solubilization in NP-40 lysis buffer that dissociated TCRζ from the CD3 components, followed by immunoprecipitations with anti-TCRζ– or anti-CD3ε–specific antibodies (data not shown) further confirmed that the phosphoproteins induced by t-DR7 represented phosphorylated ζ and those induced by t-DR7/B7-1 represented phosphorylated and hyperphosphorylated ζ and phosphorylated CD3 complex. These results demonstrate that immunogenic stimulation can activate phosphorylation of γ, δ, ε, and ζ chains of the TCR, whereas anergizing stimulation results only in partial phosphorylation of ζ chain.

After Anergizing Stimulation with Alloantigen, TCRζ Can Be an In Vitro Substrate for Activated fyn, Whereas in the Presence of B7 Costimulation, TCRζ Can Be an In Vitro Substrate for activated lck and ZAP-70. In light of our data that TCRζ was differentially phosphorylated under conditions that resulted in anergy or immunity, we examined whether specific PTK activity was also differentially associated with phosphorylated TCRζ. Alloreactive T cell clones were stimulated and anti-TCRζ immunoprecipitations were ex-

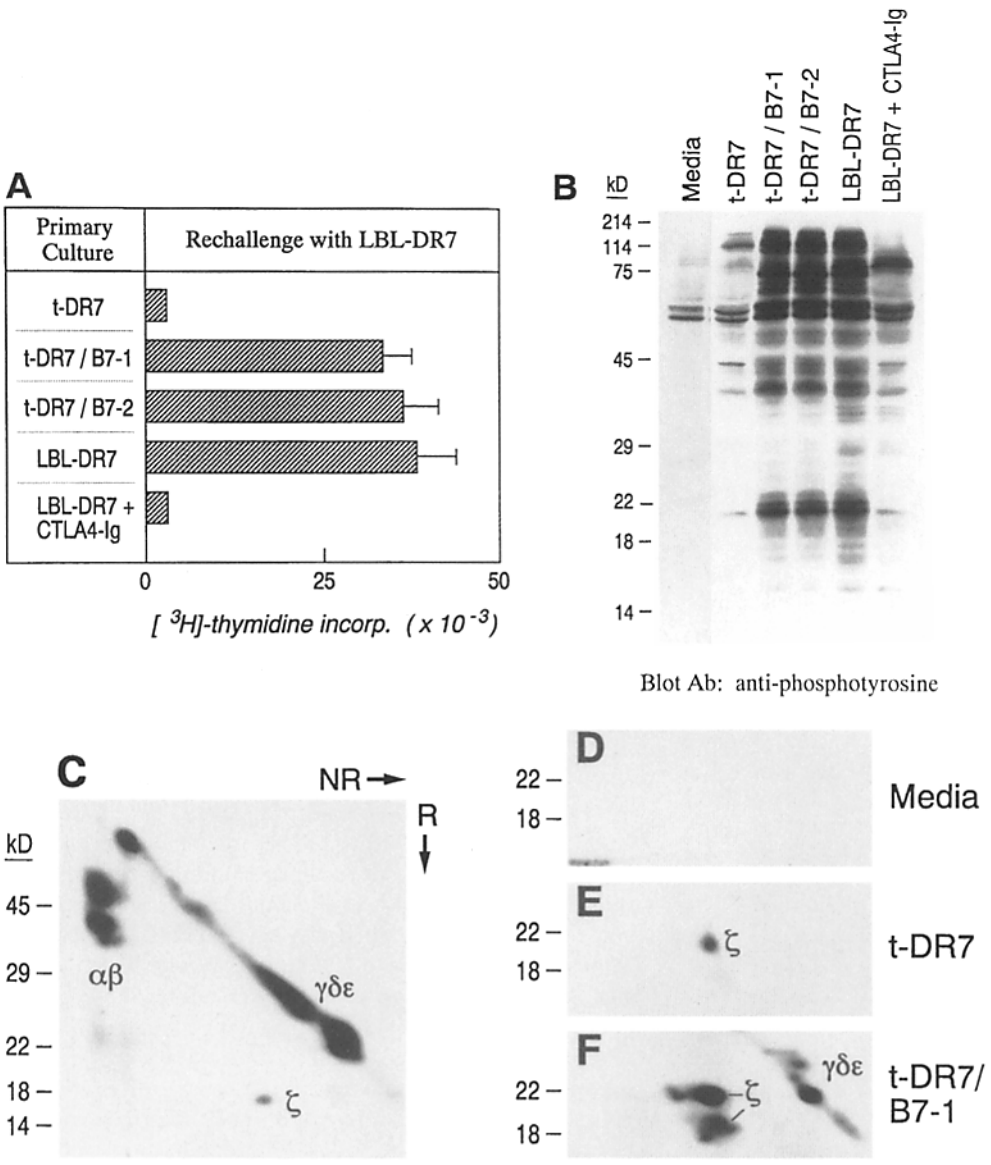

Figure 1. (*A*) TCR signaling in the absence of B7 family costimulation results in alloantigen-specific clonal anergy. TC-1 clone was primarily cultured with either t-DR7, t-DR7/B7-1, t-DR/B7-2, LBL-DR7, or LBL-DR7+CTLA4-Ig. TC-1 cells were isolated from the primary culture, rechallenged with LBL-DR7, and thymidine incorporation was measured for the last 16 of a 72-h culture period. Results are representative of 10 experiments. (*B*) Anergizing and immunogenic stimulation activate distinct patterns of protein tyrosine phosphorylation. TC-1 cells were incubated with the indicated stimulators for 10 min, and activation of protein tyrosine phosphorylation was examined in whole cell lysates by antiphosphotyrosine immunoblot. Results are representative of 15 experiments. (*C*) Structure of the TCR complex of TC-1 cells. TC-1 (2×10^7) cells were radioiodinated and solubilized with lysis buffer containing 1% digitonin; TCR chains were immunoprecipitated with anti-CD3ε mAb and analyzed on two-dimensional nonreducing (*NR*)/reducing (*R*) diagonal gel electrophoresis. Results are representative of three experiments. (*D–F*) Anergizing and immunogenic allo-APCs activate differential tyrosine phosphorylation of TCR components. TC-1 cells were cultured with (*D*) media, (*E*) t-DR7 allo-APCs, or (*F*) t-DR7/B7-1 allo-APCs for 10 min. Cell lysates were prepared with lysis buffer containing 1% digitonin. The TCR complex was immunoprecipitated with anti-CD3ε mAb, separated by two-dimensional nonreducing (*NR*)/reducing (*R*) diagonal gel electrophoresis; tyrosine-phosphorylated proteins were detected by antiphosphotyrosine immunoblot. Results are representative of five experiments.

amined for associated kinase activity by in vitro kinase reactions. Unstimulated alloreactive T cell clones contained a kinase activity constitutively associated with TCRζ that resulted in low background levels of in vitro TCRζ phosphorylation detectable only after prolonged exposure, although the associated kinase could not be seen (data not shown). After an anergizing signal, a significant increase in kinase activity associated with TCRζ could be detected. TCRζ was a substrate for in vitro tyrosine phosphorylation that resulted in the generation of 16-, 18-, and 21-kD bands. A band with a higher molecular mass of ~59–60 kD was also phosphorylated (Fig. 2 *A*). After an immunogenic signal, ζ-associated kinase(s) induced a different pattern of TCRζ phosphorylation resulting in the generation of an additional 22-kD phosphorylated band, and new substrates of ~56–60 and 70 kD were also observed (Fig. 2 *A*). The identity of the activated TCR-associated PTKs after anergizing or immunogenic stimulation was next investigated by reprecipitation experiments. fyn but not lck or ZAP-70 could be reprecipitated from ζ-immune complexes after anergizing stimulation (Fig. 2 *B*). In contrast, lck and ZAP-70, but not fyn, were detected in ζ-immune complexes after immunogenic stimulation (Fig. 2 *B*).

The nature of the additional 22-kD phosphorylated product observed after immunogenic stimulation was next examined by phosphoaminoacid analysis and by two-dimensional gel electrophoresis. Phosphoaminoacid analysis showed that both the 21- and 22-kD bands were phosphorylated on tyrosine and not serine or threonine (Fig. 2 *C*), indicating that the associated kinase activity was specific for tyrosine residues. Two dimensional nonreducing/reducing gel electrophoresis demonstrated that the 22-kD phos-

phoprotein migrated below the diagonal, confirming its identity as that of phospho-ζ (Fig. 2 *D*).

After Anergizing Stimulation with Alloantigen, There Is No Increase in CD3ϵ-associated Tyrosine Kinase Activity, Whereas in the Presence of B7 Costimulation, CD3ϵ Can Be an In Vitro Substrate for Activated fyn, lck, and ZAP-70. CD3ϵ becomes phosphorylated after T cell activation (37, 38) and has been shown to associate with PTK activity (38, 39). Since CD3ϵ was phosphorylated under immunogenic (Fig. 1 *F*) but not under anergizing conditions (Fig. 1 *E*), we sought to determine whether the lack of ϵ phosphorylation observed under anergizing culture conditions was due to the lack of as-

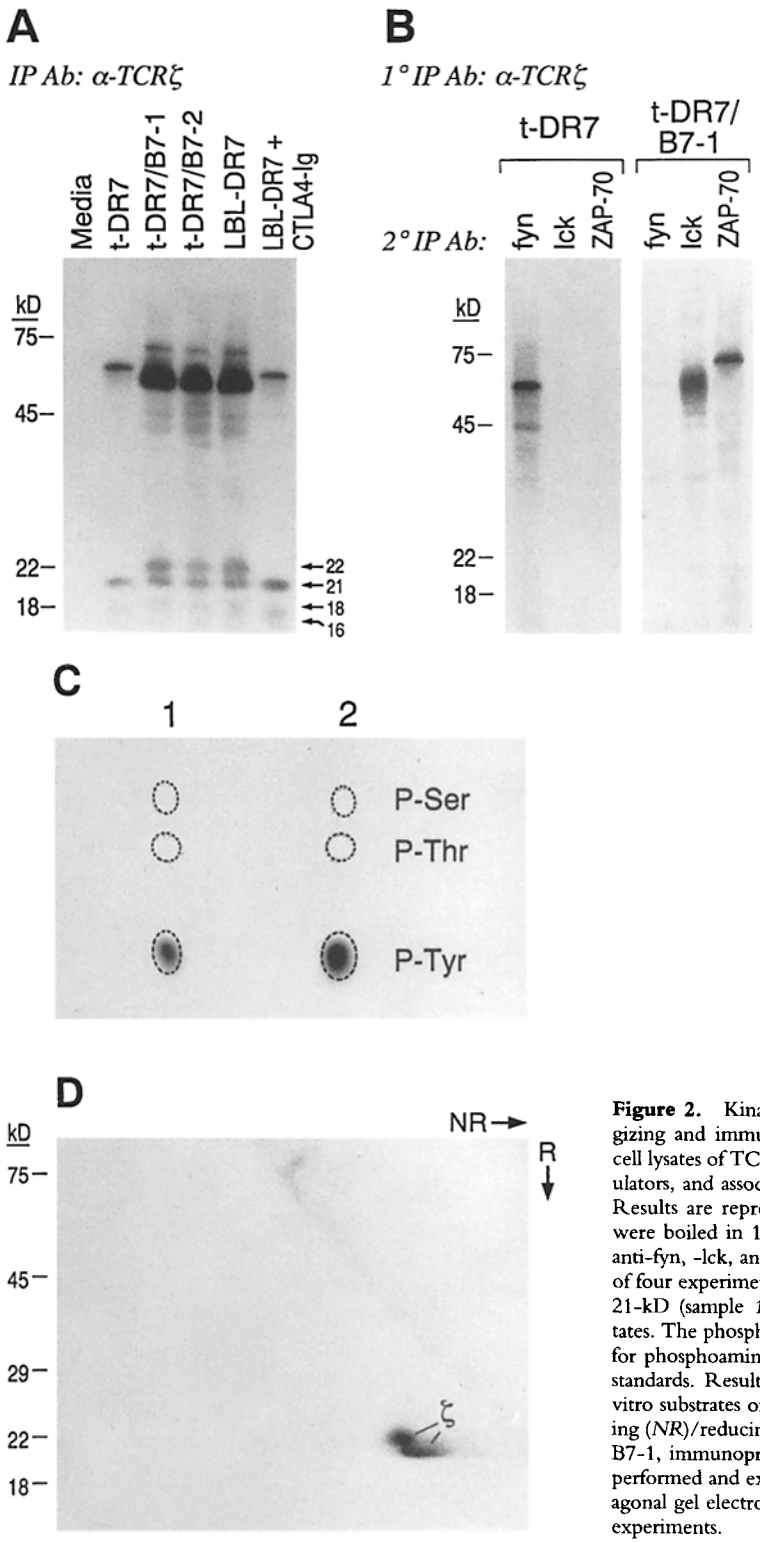

Figure 2. Kinase activity associated with TCRζ immunoprecipitates after anergizing and immunogenic stimulation. (*A*) TCRζ was immunoprecipitated from cell lysates of TC-1 (5×10^6) stimulated for 5 min at 37°C with the indicated stimulators, and associated kinase activity was examined. Exposure time was 30 min. Results are representative of six experiments. (*B*) Immunoprecipitations from *A* were boiled in 1% SDS, diluted 10-fold in lysis buffer, and reprecipitations with anti-fyn, -lck, and -ZAP-70 antiserum were performed. Results are representative of four experiments. (C) Phosphoaminoacid analysis of the in vitro phosphorylated 21-kD (sample *1*) and 22-kD (sample *2*) products from TCRζ immunoprecipitates. The phosphorylated 21- and 22-kD products were hydrolyzed and examined for phosphoaminoacid content. (*Dashed circles*) Position of the phosphoaminoacid standards. Results are representative of two experiments. (*D*) Analysis of the in vitro substrates of TCRζ-associated kinase activity by two-dimensional nonreducing (*NR*)/reducing (*R*) diagonal gel electrophoresis. After stimulation with t-DR7/B7-1, immunoprecipitations and in vitro immune complex kinase reactions were performed and examined by two-dimensional nonreducing (*NR*)/reducing (*R*) diagonal gel electrophoresis and autoradiography. Results are representative of three experiments.

sociation with activated PTKs. Nondiscernible levels of in vitro tyrosine kinase activity were observed in anti-CD3ε immunprecipitates prepared from clones cultured with media alone (Fig. 3 *A*) or control transfectants (data not shown). Interestingly, no CD3ε-associated kinase activity above background levels was observed after anergizing stimulation. In contrast, immunogenic stimulation significantly enhanced the CD3ε-associated kinase activity and generated phosphorylated products of 23–25 kD which, as previously shown (38), represent the phosphorylated subunits (γ, δ, and ε) of the CD3 complex, and additional new substrates of ~56–70 kD (Fig. 3 *A*). It has been shown that CD3ε constitutively associates with fyn PTK in a murine T cell hybridoma (40) and can be induced to associate with ZAP-70 after receptor stimulation (38). In addition, lck activity was shown to associate with CD3ε in a leukemic T cell line (HPB-ALL) (41), Jurkat, and peripheral blood T cells (42). Therefore, reprecipitation of the in vitro kinase reaction products was performed with PTK-specific antisera, which showed that fyn, lck, and ZAP-70 kinase activity were detectable in CD3ε-immune complexes after immunogenic stimulation (Fig. 2 *D*).

In Intact Cells, TCRζ Is Differentially Phosphorylated under Anergizing and Immunogenic Stimulation, Whereas CD3ε Is Tyrosine Phosphorylated Only under Immunogenic Stimulation. In vivo induced tyrosine phosphorylation of TCRζ and CD3ε and their associated kinases under anergizing and immunogenic conditions was examined by antiphosphotyrosine immunoblot, and results parallel to those obtained by in vitro kinase reactions were observed (Fig. 4). Anti-ζ immunoprecipitations from T cell clones stimulated with anergizing stimuli demonstrated that TCRζ underwent tyrosine phosphorylation resulting in a major phosphorylated band of 21 kD and a minor band of 18 kD, which as previously described, represent phosphorylated forms of TCRζ (35–37). In contrast, anti-ζ immunoprecipitation after immunogenic stimuli demonstrated the generation of two major 21- and 22-kD phosphoproteins and several minor phosphoproteins ranging from 16 to 18 kD. CD3ε became phosphorylated after immunogenic but not anergizing stimulation. Immunoblotting with a mAb that detects only the nonphosphorylated form of TCRζ, showed that the amount of nonphosphorylated TCRζ was reduced following immunogenic stimulation as compared to anergizing stimulation and unstimulated cells. However, no quantitative comparison of the amounts of phosphorylated and nonphosphorylated TCRζ could be done because of the different affinities of the anti-phosphotyrosine and anti-TCRζ mAbs. No TCRζ was detectable in the anti-CD3ε immunoprecipitations.

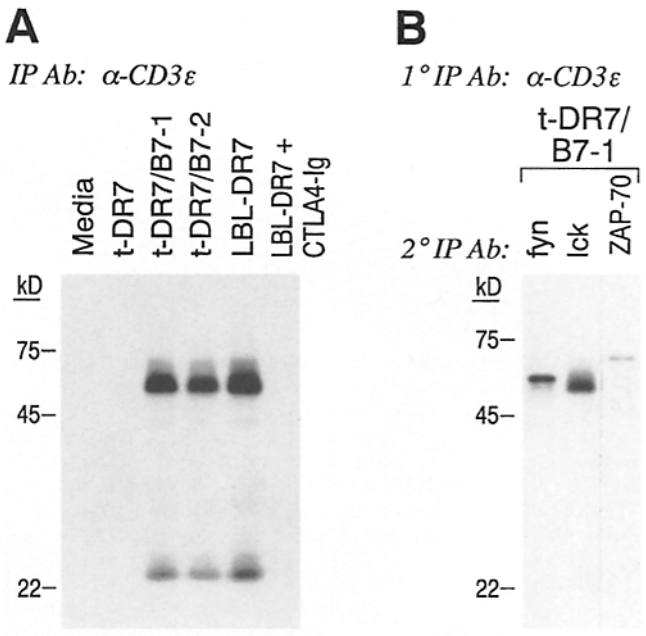

Figure 3. Kinase activity associated with CD3ε immunoprecipitates after anergizing and immunogenic stimulation. (*A*) CD3ε was immunoprecipitated from lysates of TC-1 cells (5×10^6) stimulated for 5 min at 37°C with the indicated stimulators, and associated kinase activity was examined. Exposure time was 30 min. Results are representative of four experiments. (*B*) Reprecipitation was performed as described in Fig. 2 *B*.

The identity of several TCRζ- and CD3ε-associated phosphoproteins under various culture conditions was examined by immunoblotting with PTK-specific antisera (Fig. 4), and results parallel to those obtained by reprecipitations from in vitro kinase reaction products were observed. However, no fyn was detected associated with CD3ε under anergizing or immunogenic stimulation. The observation that fyn can be detected in reprecipitation experiments as an ε-associated in vitro phosphorylated product from in vitro kinase reaction after immunogenic stimulation (Fig. 3 *B*) is probably due to the higher sensitivity of the assay.

After TCR Ligation by Alloantigen and CD28 by B7-1 or B7-2, CD28 Becomes Associated with Activated lck and TCRζ. Since t-DR7/B7-1 or t-DR7/B7-2 induced a significant increase in tyrosine phosphorylation and generated similar TCRζ- and CD3ε-associated PTK activity, we examined whether CD28 might be involved in the activation of associated kinase(s). Although CD28 immunoprecipitations from unstimulated cells or those stimulated with anergizing stimulus (t-DR7) did not demonstrate significant kinase activity, immunoprecipitations after stimulation with t-DR7/B7-1 or t-DR7/B7-2 demonstrated multiple associated phosphoproteins (Fig. 5 *A*). Reprecipitation from these immune complex kinase samples with anti-fyn, lck, and ZAP-70 antisera demonstrated that CD28 was associated with lck after immunogenic stimulation. In two of six experiments, small quantities of fyn could also be detected in the CD28-immune complexes (Fig. 5 *B*). The observation that CD28 was associated with a TCR-associated kinase under immunogenic stimulation raised the possibility that simultaneous cross-linking of TCR and CD28 by their respective ligands, might result in the physical association of TCR and CD28, so that potentially they share common PTKs. Therefore, the presence of TCRζ was examined in immune complex kinase reactions performed on CD28 immunoprecipitations after t-DR7/B7-1 or t-DR7/B7-2 stimulation. Anti-TCRζ immunoprecipitations from CD28-immune complexes after t-DR7/B7-1 or t-DR7/B7-2

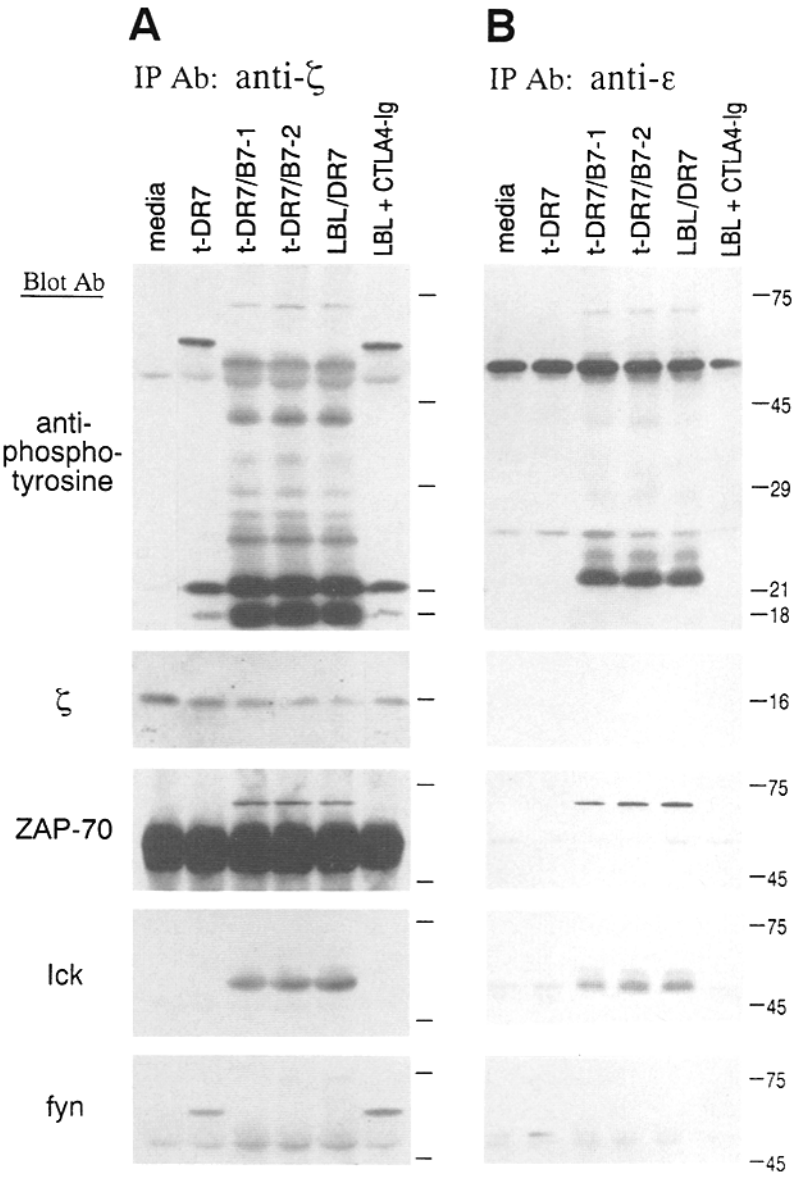

Figure 4. Association of tyrosine phosphoproteins with TCRζ and ϵ after anergizing and immunogenic stimulation. T cells were stimulated with the indicated stimulators, lysates were prepared and immunoprecipitated with anti-TCRζ (*A*) or anti-CD3ϵ (*B*) antibodies. After SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunoblotted with antiphosphotyrosine mAb, anti-TCRζ mAb, or specific antiserum for ZAP-70, lck, and fyn, followed by horseradish peroxidase anti–mouse IgG, horseradish peroxidase–protein A, or horseradish peroxidase anti–rabbit-IgG, as described in Materials and Methods. The band observed at ~50 kD represents the Ig heavy chain. In the ZAP-70 immunoblot, the heavy chain is more prominent in Fig. 3 *A* than in *B* because anti-TCRζ is a rabbit antipeptide antiserum, whereas anti-CD3ϵ is a mouse mAb. Results are representative of 12 experiments.

stimulation showed that the highly phosphorylated form of TCRζ (21/22 kD) was detected (Fig. 5 *B*).

Discussion

Ever-mounting evidence supports the notion that B7 family–mediated costimulation is critical for the induction of a successful primary immune response. TCR ligation in absence of B7 costimulation results in the induction of anergy, whereas TCR ligation accompanied by B7-mediated costimulation is sufficient to prevent anergy (18–20, 22, 43, 44). The biologic significance of this pathway has been well established in murine models, clearly demonstrating the role of B7 in the generation of autoimmunity (45–47), tumor immunity (48–51), and allograft rejection (52). Moreover, blockade of the B7:CD28 costimulatory pathway has been shown to inhibit humoral immunity (53), graft rejection (54, 55), GVHD (56), and to ameliorate autoimmune disease (57, 58). Therefore, this pathway provides great potential for the induction and prevention of immunity in a variety of clinical settings.

Although it is unclear how TCR ligation leads to initiation of tyrosine phosphorylation, fyn and lck appear to be directly implicated in the phosphorylation of the tyrosines within the immune receptor tyrosine activation motifs (ITAMs) of the TCRζ and CD3 chains (6, 38, 59). Our data show that after anergizing stimulation, fyn is associated with and can induce in vitro phosphorylation of TCRζ but not CD3ϵ. In contrast, after immunogenic stimulation, lck is associated with and can induce phosphorylation of TCRζ and ϵ chains, resulting in recruitment and association of ZAP-70. Since fyn is constitutively associated with both ζ and CD3 chains but has higher affinity for ζ (60), our results suggest that an anergizing stimulus can trigger fyn acti-

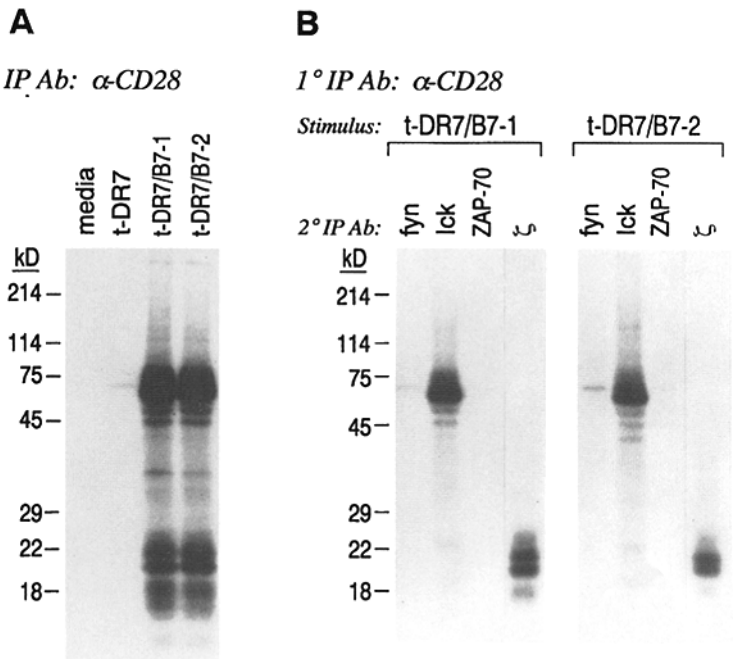

Figure 5. Association of CD28 with lck and hyperphosphorylated TCRζ after simultaneous ligation of TCR and CD28. (*A*) TC-1 cells (5×10^6) were cultured with either media, t-DR7, t-DR7/B7-1, or t-DR7/B7-2, for 5 min and associated kinase activity in CD28 immunoprecipitates was examined. (*B*) After stimulation with t-DR7/B7-1 or t-DR7/B7-2 and anti-CD28 immunoprecipitations, reprecipitations with anti-fyn, -lck, -ZAP-70 or -TCRζ antiserum were performed as in Fig. 2 *B*. Results are representative of six experiments.

vation sufficient to phosphorylate ζ but not γ, δ, or ϵ. Preliminary results from our laboratory show that fyn binding to the partially phosphorylated TCRζ after anergizing stimulation is mediated via its SH2 domain. This finding suggests that fyn, which is constitutively associated with TCR via its NH_2-terminal site, may be the first kinase to initiate TCR phosphorylation. After receptor phosphorylation, reorientation of fyn occurs which now associates with the receptor via its SH2 domain. Similar observations regarding reorientation of protein kinases on receptor-binding sites have been reported in different systems (61).

Alloantigen binding under anergizing conditions induces fyn activation, generation of partially phosphorylated TCRζ (18 and 21 kD) and downstream-associated substrates. These events result in partial but not complete functional outcome of the immune response, characterized by upregulation of cytokine receptors and induction of low levels of IL-2 mRNA but no proliferation or cytokine accumulation. If B7 costimulation is available, additional activation events are induced resulting in further phosphorylation of the TCR/CD3 complex. Under these conditions, lck also becomes activated and associates with TCRζ and ϵ. Although ζ can serve as in vitro substrate and become phosphorylated by lck (41), Gauen et al. (60) have shown that lck cannot associate with nonphosphorylated ζ. Therefore, fyn may initiate and lck may optimize phosphorylation of the receptor. Fully phosphorylated receptor now recruits ZAP-70 to bind via its dual SH2 domains. Partial phosphorylation of TCRζ after an anergizing signal can generate phosphorylated sites adequate for fyn-SH2 binding but inadequate for ZAP-70 binding, which, because of its structure, requires binding of both its SH2 domains on phosphorylated substrates, indicating that ZAP-70 binds only on doubly phosphorylated ITAMs (4, 62–65) .

Our data suggest that the induction of anergy is an active signaling process characterized by the association of TCRζ and fyn. Whether this is the consequence of specific fyn-mediated events, or alternatively, the consequence of inadequate activation of lck and ZAP-70 and absence of their associated biochemical events, remains to be determined. It is of note that in two distinct, costimulation-independent systems in which anergy is induced in vitro by altered peptide ligands (66, 67) or in vivo by superantigen (68), partial TCRζ phosphorylation and absence of ZAP-70 activation was reported, underscoring the more general importance of this observation in the generation of anergy. Evidence for the association of fyn with the state of anergy is less well developed. However, Quill et al. (69) observed that anergic cells expressed higher total cellular levels of fyn and decreased levels of lck compared with unstimulated or productively stimulated murine T cell clones. In another murine model, increased fyn kinase activity was detected in anergized cells compared with productively stimulated or unstimulated cells (70). In a human system, the association of fyn activation with a state of antigen-specific unresponsiveness is supported by a recent study (71) demonstrating that $CD4^+$ T cells from early HIV infection states are characterized by unresponsiveness to TCR-mediated stimulation and exhibit high levels of fyn and low levels of lck activity. Taken together with the above results, our data suggest that fyn and lck do not have redundant functions in early T cell activation. Consistent with this hypothesis is the observation that lck mutants, which demonstrate levels of fyn kinase comparable with their wild-type counterparts, fail to activate tyrosine phosphorylation or to express activation antigens after TCR stimulation (9). Genetic evidence derived from fyn- and lck-deficient mice also suggest that the in vivo physiologic roles of these tyrosine kinases

do not overlap. The lck-deficient mouse demonstrates a highly significant defect in thymic maturation and the few remaining mature T lymphocytes are hyporesponsive to TCR-mediated signals (7, 72). This profound defect argues that the alternative src kinase fyn cannot substitute for lck. In contrast, fyn-deficient mice are relatively immunologically intact and the only observed defect is the reduced capacity of mature thymocytes to respond to TCR stimulation (2, 8). Moreover, functional antigen-specific clones have been successfully generated from these fyn-deficient mice (73).

CD28 does not merely enhance the magnitude of TCR signal transduction but rather mediates activation of a distinct signaling pathway. CD28-mediated signaling is resistant to cyclosporin A (74), generates novel lipids different from those generated by TCR cross-linking (75), and activates a specific DNA-binding complex that interacts with cytokine promoters (76, 77). As shown here, in the presence of an anergizing stimulation, CD28 ligation results in productive functional outcome characterized by IL-2 accumulation, proliferation, activation of a distinct pattern of tyrosine phosphorylation of TCR and cytoplasmic proteins, and association of TCR with lck and ZAP-70. Therefore, although the downstream pathways of TCR and CD28 are distinct, CD28 cross-linking, independent of its downstream events, modifies the initial TCR signal. Several groups have reported that CD28 cross-linking results in a low but consistent lck activation (78–80). Although lck activation in TCR-mediated stimulation is obligatory (9), its significance on the CD28 mediated downstream events is still unclear. It has been suggested that PI-3 kinase binding to CD28, as well as grb2/sos and ITK binding is dependent upon CD28 phosphorylation by src kinases, most notably lck (81). However, stimulation of a lck-defective cell line with PMA and ionomycin in the presence or absence of CD28 resulted in IL-2 secretion similar to that seen in the wild type, suggesting that lck does not play an obligatory role in CD28 downstream signaling (82). Therefore, CD28-mediated lck activation may not have a functional role on the CD28 downstream signaling, but rather, may be important for the initiation of a successful TCR signal.

Although CD28 can associate with src family kinases after cross-linking, CD28 does not have a constitutively associated kinase activity similar to CD4 or CD8. However, many recognition events in the immune system are initiated by aggregation of cell surface receptors that lack intrinsic protein kinase activity. There is increasing evidence that src family kinases can associate with a number of cell surface molecules that are not members of the antigen receptor family, including CD2, CD23, CD36, IL-2Rβ chain, and various phosphatidylinositol-anchored proteins, some of which also require simultaneous ligation of the antigen receptor to promote activation (83–88). A potential explanation for this requirement is that the triggering motif on the antigen receptor acts as substrate for these src kinases allowing subsequent recruitment of ZAP kinase (89). Aggregation-induced repartitioning of enzyme and substrate may be an important factor in redirecting an existing activity towards the appropriate target. Indeed, our results show that after TCR and CD28 ligation by antigen and B7 respectively, lck becomes associated not only with CD28 but also with TCRζ.

An intriguing explanation for the requirement of simultaneous ligation of TCR and CD28 to induce the above discussed biochemical events and functional outcome is that ligation results in redistribution of the surface molecules in the T:APC contact patch so that TCR/CD3 and CD28 become components of a molecular complex. Under these circumstances, lck becomes activated and associates with both CD28 and TCR, resulting in sufficient tyrosine phosphorylation of the ITAMs and successful initiation of the TCR-associated downstream signaling events. The previous observation that both antigen and B7 have to be expressed on the same APC to induce an optimal response (20, 90) is consistent with this idea. The superior ability of ligand and costimulator coexpressed on the same APC to activate T cells suggests that the signals delivered by the TCR and the costimulatory molecule must be integrated at or near the T cell surface membrane that is in contact with the APC (91). Whether additional molecules that may activate associated lck are recruited to the complex remains to be determined.

This work has been supported by National Institutes of Health grants CA-40216, AI-35225, AL-54785, and by the Alberta Heritage Foundation for Medical Research (D.L. Barber).

Address correspondence to Dr. Vassiliki A. Boussiotis, Dana-Farber Cancer Institute, Dana 740, 44 Binney Street, Boston, MA 02115.

Received for publication 5 March 1996 and in revised form 8 May 1996.

References

1. Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263–274.
2. Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Quian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of $p59^{fyn}$. *Cell.* 70:751–763.

3. Chan, A.C., B.A. Irving, J.D. Fraser, and A. Weiss. 1991. The ζ chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proc. Natl. Acad. Sci. USA.* 88:9166–9170.
4. Chan, S.H., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: A 70kD protein-tyrosine kinase that associates with TCRζ chain. *Cell.* 71:649–662.
5. Glaichenhaus, N., N. Shastri, D.R. Littman, and J.M. Turner. 1991. Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. *Cell.* 64:511–520.
6. Karnitz, L., S.L. Sutor, T. Torigoe, J.C. Reed, M.P. Bell, D.J. McKean, P.J. Leibson, and R.T. Abraham. 1992. Effects of $p56^{lck}$ deficiency on the growth and cytolytic effector function of an interleukin-2–dependent cytotoxic T-cell line. *Mol. Cell. Biol.* 12:4521–4530.
7. Molina, T.J., K. Kishihara, D.P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C.J. Paige, K.U. Hartmann, A. Veillette et al. 1992. Profound block in thymocyte development in mice lacking $p56^{lck}$. *Nature (Lond.).* 357:161–164.
8. Stein, P.L., H.M. Lee, S. Rich, and P. Soriano. 1992. $p59^{fyn}$ mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell.* 70:741–750.
9. Straus, D., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell.* 70:585–593.
10. 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 antigen receptor occupancy. *Annu. Rev. Immunol.* 7: 445–480.
11. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302–319.
12. Jenkins, M.K., J.D. Ashwell, and R.H. Schwartz. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol.* 140:3324–3330.
13. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J. Immunol.* 142:2617–2628.
14. 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.* 143:2714–2722.
15. 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: 721–730.
16. Freeman, G.J., J.G. Gribben, V.A. Boussiotis, J.W. Ng, V. Restivo, L. Lombard, G.S. Gray, and L.M. Nadler. 1993. Cloning of B7-2: a CTLA4 counter–receptor that costimulates human T cell proliferation. *Science.* 262:909–911.
17. Azuma, M., D. Ito, H. Yagita, K. Okumura, J.H. Phillips, L.L. Lanier, and C. Somoza. 1993. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature (Lond.).* 366:76–79.
18. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (Lond.).* 356:607–609.
19. Gimmi, C.D., G.J. Freeman, J.G. Gribben, G. Gray, and L.M. Nadler. 1993. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc. Natl. Acad. Sci. USA.* 90:6586–6590.
20. Boussiotis, V.A., G.J. Freeman, G. Gray, J. Gribben, and L.M. Nadler. 1993. B7 but not ICAM-1 costimulation prevents the induction of human alloantigen specific tolerance. *J. Exp. Med.* 178:1753–1763.
21. Essery, G., M. Feldmann, and J. Lamp. 1988. Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. *Immunology.* 64:413–417.
22. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvard, J. Bradshaw, J.A. Ledbetter, and P. Linsley. 1993. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* 177:165–173.
23. Boussiotis, V.A., D.L. Barber, T. Nakarai, G.J. Freeman, J.G. Gribben, G.M. Bernstein, A.L. D'Andrea, J. Ritz, and L.M. Nadler. 1994. Prevention of T cell anergy by signaling through the γc chain of the IL-2 receptor. *Science (Wash. DC).* 266:1039–1042.
24. Boussiotis, V.A., G.J. Freeman, J.D. Griffin, G.S. Gray, J.G. Gribben, and L.M. Nadler. 1994. CD2 is involved in maintenance and reversal of human alloantigen specific clonal anergy. *J. Exp. Med.* 180:1665–1673.
25. Freeman, G.J., V.A. Boussiotis, A. Anumanthan, G.M. Bernstein, X.-Y. Ke, P.D. Rennert, G.S. Gray, J.G. Gribben, and L.M. Nadler. 1995. B7-1 and B7-2 do not deliver identical costimulatory signals since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity.* 2:523–532.
26. Anderson, P., M.L. Blue, C. O'Brien, and S.F. Schlossman. 1989. Monoclonal antibodies reactive with the T cell receptor ζ chain: production and characterization using a new method. *J. Immunol.* 143:1899–1904.
27. Transy, C., P.E. Moingeon, B.C. Marshall, S. Schlossman, and E.L. Reinherz. 1989. Most anti-human CD3 monoclonal antibodies are directed to the CD3 epsilon subunit. *Eur. J. Immunol.* 19:947–950.
28. Philips, D.R., and M. Morrison. 1971. Exposed protein of the intact human erythrocyte. *Biochemistry.* 10:1766–1771.
29. Barber, D., and A. D'Andrea. 1994. Erythropoietin and interleukin-2 activate distinct JAK kinase family members. *Mol. Cell. Biol.* 14:6506–6514.
30. Rudd, C.E., J.M. Trevillyan, J.D. Dasgupta, L.L. Wong, and S.F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:5190–5194.
31. Ernst, T., K.E. Slattery, and J.D. Griffin. 1994. $p210^{Bcr/Abl}$ and $p160^{v\text{-}Abl}$ induce an increase in the tyrosine phosphorylation of $p93^{c\text{-}Fes}$. *J. Biol. Chem.* 269:5764–5769.
32. 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.* 144:1591–1599.
33. Klausner, R.D., J.J. O'Shea, H. Luong, P. Ross, J. Bluestone, and L.E. Samelson. 1987. T cell receptor tyrosine phosphorylation. Variable coupling for different activating ligands. *J. Biol. Chem.* 262:12654–12659.

34. Weissman, A.M., P. Ross, E.T. Luong, P. Garcia-Morales, M.L. Jelachich, W. Biddison, R.D. Klausner, and L.E. Samelson. 1988. Tyrosine phosphorylation of the human T cell antigen reseptor ζ chain: activation via CD3 but not CD2. *J. Immunol.* 141:3532–3536.
35. Koyasu, S., D.J. McConkey, L.K. Clayton, S. Abraham, B. Yandava, T. Katagiri, P. Moingeon, T. Yamamoto, and E.L. Reinherz. 1992. Phosphorylation of multiple CD3ζ tyrosine residues leads to formation of pp21 in vitro and in vivo. *J. Biol. Chem.* 267:3375–3381.
36. Baniyash, M., P. Garcia-Morales, E. Luong, L.E. Samelson, and R.D. Klausner. 1988. The T cell antigen receptor ζ chain is tyrosine phosphorylated upon activation. *J. Biol. Chem.* 263:18225–18230.
37. Quian, D., I. Griswold-Prenner, M. Rich Rosner, and F.W. Fitch. 1993. Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation. *J. Biol. Chem.* 268:4488–4493.
38. Straus, D.B., and A. Weiss. 1993. The CD3 chains of the T cell antigen receptor associate with ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. *J. Exp. Med.* 178:1523–1530.
39. 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.* 145:2448–2454.
40. Samelson, L.E., A.F. Phillips, E.T. Luong, and R.D. Klausner. 1990. Association of the fyn protein-tyrosine kinase with the T cell antigen receptor. *Proc. Natl. Acad. Sci. USA.* 87: 4358–4362.
41. Burgess, K.E., A. Odysseos, A.D. Zalvan, B. Drucker, P. Anderson, S.F. Schlossman, and C.E. Rudd. 1991. Biochemical identification of a direct physical interaction between the CD4:p56^{lck} and ti(TCR)/CD3 complexes. *Eur. J. Immunol.* 21:1663–1668.
42. Burgess, K.E., M. Yamamoto, K.V.S. Prasad, and C.E. Rudd. 1992. CD5 acts as a tyrosine kinase substrate within a receptor complex comprising T-cell receptor ζ chain/CD3 and protein-tyrosine kinases p56lck and p59fyn. *Proc. Natl. Acad. Sci USA.* 89:9311–9315.
43. Young, J.W., L. Koulova, S.A. Soergel, E.A. Clark, R.M. Steinman, and B. Dupont. 1992. The B7/BB1 antigen provides one of several costimulatory signals for the activation of CD4$^+$ T lymphocytes by human blood dendritic cells in vitro. *J. Clin. Invest.* 90:229–237.
44. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB1 provides a costimulatory signal for alloactivation of CD4$^+$ T cells. *J. Exp. Med.* 173:759–762.
45. Guerder, S., J. Meyerhoff, and R. Flavell. 1994. The role of the T cell costimulator B7-1 in autoimmunity and the induction and maintenance of tolerance to peripheral antigen. *Immunity.* 1:155–166.
46. Harlan, D.M., H. Hengartner, M.L. Huang, Y.H. Kang, R. Abe, R.W. Moreadith, H. Pircher, G.S. Gray, P.S. Ohashi, G.J. Freeman et al. 1994. Transgenic mice expressing both B7 and viral glycoprotein on pancreatic beta cells along with glycoprotein-specific transgenic T cells develop diabetes due to a breakdown of T lymphocyte unresponsiveness. *Proc. Natl. Acad. Sci. USA.* 91:3137–3141.
47. Verwilghen, J., R. Lovis, M. De Boer, P. Linsley, G. Haines, A. Koch, and R. Pope. 1994. Expression of functional B7 and CTLA4 on rheumatoid synovial T cells. *J. Immunol.* 153: 1378–1385.
48. Chen, L., A. Ashe, W.A. Brady, I. Hellstrom, K.E. Hellstrom, J.A. Ledbetter, P. McGowan, and P. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecule CD28 and CTLA-4. *Cell.* 71:1093–1102.
49. Townsend, S.E., and J.P. Allison. 1993. Tumor rejection after direct costimulation of CD8$^+$ T cells by B7-transfected melanoma cells. *Science (Wash. DC).* 259:368–370.
50. Ramarathian, L., M. Castle, Y. Wu, and Y. Liu. 1994. T cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment and effector function of antitumor T cells. *J. Exp. Med.* 179:1205–1214.
51. Matulonis, U., C. Dosiou, C. Lamont, G.J. Freeman, P. Mauch, L.M. Nadler, and J.D. Griffin. 1995. Role of B7-1 in mediating an immune response to myeloid leukemia cells. *Blood.* 85:2507–2515.
52. Turka, L.A., P.S. Linsley, H. Lin, W. Brady, J.M. Leiden, R.Q. Wei, M.L. Gibson, X.G. Zheng, S. Myrdal, D. Gordon et al. 1992. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc. Natl. Acad. Sci. USA.* 89:11102–11105.
53. Linsley, P.S., P.M. Wallace, J. Johnson, M.G. Gibson, J.L. Greene, J.A. Ledbetter, C. Singh, and M.A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science (Wash. DC).* 257:792–795.
54. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S. Linsley, and J.A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science (Wash. DC).* 257:789–792.
55. Lin, H., S.F. Bolling, P. Linsley, R.Q. Wei, D. Gordon, C.A. Thompson, and L.A. Turka. 1993. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J. Exp. Med.* 178:1801–1806.
56. Blazar, B.R., P.A. Taylor, P.S. Linsley, and P.A. Vallera. 1994. In vivo blockade of CD28/CTLA4: B7/BB1 interaction with CTLA4-Ig reduces lethal murine graft-versus-host disease across the major histocompatibility complex barrier in mice. *Blood.* 83:3815–3825.
57. Finck, B., P. Linsley, and D. Wofsy. 1994. Treatment of murine lupus with CTLA4Ig. *Science (Wash. DC).* 265:1225–1227.
58. Milich, D., P. Linsley, J. Hughes, and J. Jones. 1994. Soluble CTLA-4 can suppress autoantibody production and elicit long term responsiveness in a novel transgenic model. *J. Immunol.* 153:429–435.
59. Tsygankov, A.Y., C. Spana, B.R. Rowley, R.C. Penhallow, A.L. Burkhardt, and J.B. Bolen. 1994. Activation-dependent tyrosine phosphorylation of fyn-associated proteins in T lymphocytes. *J. Biol. Chem.* 269:7792–7800.
60. Gauen, L.K.T., A.N.T. Kong, L.E. Samelson, and A.S. Shaw. 1992. p59fyn tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. *Mol. Cell. Biol.* 12:5438–5446.
61. Pleinman, C.M., C. Abrahams, L.T. Gauen, W. Bedzyk, J. Jongstra, A.S. Shaw, and J.C. Cambier. 1994. Distinct p53/56^{lyn} and p59^{fyn} domains associate with nonphosphorylated and phosphorylated Ig-α. *Proc. Natl. Sci. USA.* 91:4268–4272.
62. Wange, R.L., S.N. Malek, S. Desiderio, and L.E. Samelson. 1993. Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor ζ and CD3ε from activated Jurkat T cells. *J.*

Biol. Chem. 268:19797–19801.

63. Iwashima, M., B. Irving, N. van Oers, A. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science (Wash. DC).* 263: 1136–1139.
64. Koyasu, S., A.G. Tse, P. Moingeon, R.E. Hussey, A. Mildonian, J. Hannisian, L.K. Clayton, and E.L. Reinherz. 1994. Delineation of a T-cell activation motif required for binding of protein tyrosine kinases containing tandem SH2 domains. *Proc. Natl. Sci. Acad. USA.* 91:6693–6697.
65. Hatada, M.H., X. Lu, E.R. Laird, J. Green, J.P. Morgenstern, M. Lou, C.S. Marr, T.B. Phillips, M.K. Ram, K. Theriault et al. 1995. Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. *Nature (Lond.).* 377:32–38.
66. Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen. 1994. Partial T cell signaling: altered phospho-ζ and lack of Zap70 recruitment in APL-induced T cell anergy. *Cell.* 79:913–922.
67. Madrenas, J., R.L. Wange, J.R. Wange, N. Isakov, L.E. Samelson, and R.N. Germain. 1994. ζ phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science (Wash. DC).* 267:515–518.
68. Migita, K., K. Eguchi, Y. Kawabe, T. Tsukada, Y. Ichinose, and S. Nagataki. 1995. Defective TCR-mediated signaling in anergic T cells. *J. Immunol.* 155:5083–5087.
69. Quill, H., M.P. Riley, E.A. Cho, J.E. Casnellie, J.C. Reed, and T. Torigoe. 1992. Anergic Th1 cells express altered levels of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. *J. Immunol.* 149:2887–2893.
70. Gajewski, T.F., D. Qian, P. Fields, and F.W. Fitch. 1994. Anergic T-lymphocyte clones have altered inositol phosphate, calcium and tyrosine kinase signaling pathways. *Proc. Natl. Acad. Sci. USA.* 91:38–42.
71. Cayota, A., F. Vuillier, J. Siciliano, and G. Dighiero. 1994. Defective protein tyrosine phosphorylation and altered levels of $p59^{fyn}$ and $p56^{lck}$ in CD4 T cells from HIV-1 infected patients. *Int. Immunol.* 6:611–621.
72. Levin, S.D., S.J. Anderson, K.A. Forbush, and R.M. Perlmutter. 1993. A dominant-negative transgene defines a role for $p56^{lck}$ in thymopoiesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1671–1680.
73. Lancki, D., D. Qian, P. Fields, T. Gajewski, and T.W. Fitch. 1995. Differential requirements for tyrosine kinase fyn in the functional activation of antigen-specific T lymphocytes clones through TCR or Thy-1. *J. Immunol.* 155:4363–4370.
74. 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 cyclosporin-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7:4472–4481.
75. Ward, S.G., J. Westwick, N.D. Hall, and D.M. Sansom. 1993. Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. *Eur. J. Immunol.* 23:2572–2577.
76. Fraser, J.D., and A. Weiss. 1992. Regulation of T-cell lymphokine gene transcription by the accessory molecule CD28. *Mol. Cell. Biol.* 12:4357–4363.
77. Fraser, J.D., M.E. Newton, and A. Weiss. 1992. CD28 and T cell antigen receptor signal transduction coordinately regulate interleukin 2 gene expression in response to superantigen stimulation. *J. Exp. Med* 175:1131–1134.
78. August, A., S. Gibson, K. Kawakami, T. Kawakami, G.B. Mills, and B. Dupont. 1994. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proc. Natl. Acad. Sci. USA.* 91:9347–9351.
79. August, A., and B. Dupont. 1994. Activation of src family kinase lck following CD28 crosslinking in the Jurkat leukemic cell line. *Biochem. Biophys. Res.* 199:1466–1473.
80. Hutchcroft, J.E., and B.E. Bierer. 1994. Activation-dependent phosphorylation of the T-lymphocyte surface receptor CD28 and associated proteins. *Proc. Natl. Acad. Sci. USA.* 91: 3260–3264.
81. Raab, M., Y.C. Cai, S.C. Bunnell, S.D. Heyeck, L.J. Berg, and C.E. Rudd. 1995. $p56^{lck}$ and $p59^{fyn}$ regulate CD28 binding to phosphotidylinositol 3-kinase, growth factor receptor–bound protein GRB-2, and T cell–specific protein tyrosine kinase ITK: implications for T cell costimulation. *Proc. Natl. Acad. Sci. USA.* 92:8891–8895.
82. Stein, P., J. Fraser, and A. Weiss. 1994. The cytoplasmic domain of CD28 is both necessary and sufficient for costimulation of IL-2 secretion and association with phosphatidylinositol 3′-kinase. *Mol. Cell. Biol.* 14:3392–3402.
83. Danelian, S., R. Fagard, A. Alcover, O. Acuto, and S. Fisher. 1991. The tyrosine kinase activity of $p56^{lck}$ is increased in human T cells activated via CD2. *Eur. J. Immunol.* 269:1225–1227.
84. Bell, G.M., J.B. Bolen, and J.B. Imboden. 1992. Association of src-like protein tyrosine kinases with the CD2 cell surface molecule in rat T lymphocytes and natural killer cells. *Mol. Cell. Bol.* 12:5548–5554.
85. Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S.D. Levin, R.M. Perlmutter, and T. Taniguchi. 1991. Interaction of the IL-2 receptor with the src-family kinase p56lck: identification of novel intermolecular association. *Science (Wash. DC).* 252:1523–1528.
86. Huang, M.M., Z. Indik, L.F. Brass, J.A. Hoxie, A.D. Schreiber, and J.S. Brugge. 1992. Activation of Fc gamma RII induces tyrosine phosphorylation of multiple proteins inducing Fc gamma RII. *J. Biol. Chem.* 267:5467–5473.
87. Sugie, K., T. Kawakami, M. Y., T. Kawabe, A. Uchida, and J. Yodoi. 1991. Fyn tyrosine kinase associated with Fc epsilon RII/CD23: possible multiple roles in lymphocyte activation. *J. Exp. Med.* 178:1523–1530.
88. Thomas, P.M., and L.E. Samelson. 1992. The glycophosphatidylinositol-anchored Thy-1 molecule interacts with the $p60^{fyn}$ protein tyrosine kinase in T cells. *J. Biol. Chem.* 267: 12317–12322.
89. Kolanus, W., C. Romeo, and B. Seeds. 1993. T cell activation by clustered tyrosine kinases. *Cell.* 74:171–183.
90. Liu, Y., and C. Janeway, Jr. 1992. Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. *Proc. Natl. Acad. Sci. USA.* 89:3845–3849.
91. Janeway, C.A., and K. Bottomly. 1994. Signals and signs for lymphocyte receptors. *Cell.* 76:275–285.