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

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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 CD3ζ 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), protein phosphorylation, and increased intracellular Ca2+ 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, CD3ζ, and CD28. In the results to be reported below, we demonstrate that during the induction of anergy, although protein tyrosine phosphorylation is activated, CD3ζ is not phosphorylated whereas TCRζ is only partially phosphorylated and associates with fyn. In contrast, during the induction of productive immu-
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 homoygous 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 antisemum (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 μg/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, LDL-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 density centrifugation, and cultured in media without IL-2 for 24 h. Each population was subsequently rechallenged with LDL-DR7 at a 1:1 stimulator/responder ratio. Before use, LDL-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-3H] 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 NaN3, 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 Na3PO4, 50 mM NaF, 1 mM NaN3, 200 μg/ml 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 × 108 T cell equivalents/lane) were analyzed on 12.5% gels by SDS-PAGE. Immunoprecipitations (107 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 relabelling 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 (1:5,000) 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 Na3PO4, 50 mM NaF, 1 mM NaN3, 200 μg/ml 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 Na3PO4, 50 mM NaF, 1 mM NaN3, 200 μg/ml 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 immunoblots.

Cell Surface Radioiodination and Immunoprecipitations. Cells were washed, resuspended in 200 μl cold PBS, and labeled with 1251 (1 μCi per sample) by the lactoperoxidase method (28). After washing three times in PBS to remove free iodide, cells were solubilized in digitonin lysis buffer preclotted 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/ml) 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/cond) were conducted with anti-TCRγ, anti-CD3ε, or anti-CD28 (2 μg/sample) mAbs, 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, 50 mM NaH2PO4, 50 mM NaF, 1 mM Na2VO4, aprotinin [5 μg/ml], pepstatin [1 μg/ml], soybean trypsin inhibitor [2 μg/ml] 1 mM PMSF, 0.5% Brj 96, and 0.5% NP-40) and once in kinase buffer (20 mM Heps, pH 7.2, 5 mM MgCl2, and 5 mM MnCl2). The immunoprecipitates were then suspended in 40 μl kinase buffer containing 10 μCi of γ-[32P]ATP and incubated at room temperature for 15 min. Reactions were terminated by the addition of 10 mM Heps, 10 mM EDTA, washed twice with double distilled H2O, 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 anergerizing 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 TCRγ 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 125I 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 ζ 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 anergerizing stimulation results only in partial phosphorylation of ζ chain.

**After Anergizing Stimulation with Alloantigen, TCRγ Can Be an In Vitro Substrate for Activated lyn, 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 X 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–I) 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.

Examined 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 σ-immune complexes after anergizing stimulation (Fig. 2 B). In contrast, lck and ZAP-70, but not fyn, were detected in σ-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. 2D).

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 × 10⁶) 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 immunoprecipitates 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 subunits 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, TCRε Is Differentially Phosphorylated under Anergizing and Immunogenic Stimulation, Whereas CD3ε Is Tyrosine Phosphorylated Only under Immunogenic Stimulation. In vivo induced tyrosine phosphorylation of TCRε 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-ε immunoprecipitations from T cell clones stimulated with anergizing stimuli demonstrated that TCRε 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 TCRε (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.

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 kinases(s). Although CD28 immunoprecipitations from unstimulated cells or those stimulated with anergizing stimuli (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 antiseras 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
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 energizing 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 x 10⁶) 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, reprécipitations with anti-fyn, -lck, -ZAP-70 or -TCRζ antiserum were performed as in Fig. 2 B. Results are representative of six experiments.

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. Genetic evidence derived from fyn- and lck-deficient mice also support 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 energizing 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 TCRγ.

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.

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