Syk and ZAP-70 Mediate Recruitment of p56^{lck}/CD4 to the Activated T Cell Receptor/CD3/ζ Complex

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Summary

During antigen recognition by T cells, CD4 and the T-cell receptor (TCR)/CD3/ζ complex are thought to interact with the same major histocompatibility complex II molecule in a stable ternary complex. Evidence has suggested that the association of CD4 with TCR/CD3/ζ requires the interaction of the protein tyrosine kinase p56^{lck} with CD4. We have taken a biochemical approach to understand the mechanism by which p56^{lck} and, in particular, its src homology (SH) 2 domain contributes to the association of CD4 with TCR/CD3/ζ during activation. We have previously shown that the p56^{lck} SH2 domain binds directly to tyrosine-phosphorylated ZAP-70. Here we formally demonstrate the in vivo association of p56^{lck} with the homologous protein tyrosine kinases Syk and ZAP-70 after CD3 stimulation of Jurkat cells. A tyrosine-phosphorylated peptide containing the sequence predicted to be optimal for binding to the SH2 domain of src family kinases specifically competes for this association, indicating that tyrosine-phosphorylated ZAP-70 and Syk bind to p56^{lck} by an SH2-mediated interaction. We also show that the same peptide is able to compete for the activation-dependent TCR/CD4 association in Jurkat cells. Moreover, ZAP-70 and CD4 cocap only after CD3 stimulation in human T lymphoblasts. We propose that the interaction of the p56^{lck} SH2 domain with ζ-associated tyrosine-phosphorylated ZAP-70 and/or Syk enables CD4 to associate with antigen-stimulated TCR/CD3/ζ complexes.

E ngagement of the TCR by antigen bound to MHC molecules activates a protein tyrosine kinase (PTK)[1] pathway, resulting in tyrosine phosphorylation of a number of cellular proteins (1). At least four PTKs have been shown to be implicated in this process: two kinases of the src family (p56^{lck} and p59^{fyn}) (2-6) and two of the Syk family (ZAP-70 and Syk) (7-13). Their respective contributions to TCR signaling, however, are not precisely understood.

The current model of antigen/MHC-induced T cell activation is based on a sequential interaction of these PTKs with the TCR/CD3/ζ complex, which by itself lacks intrinsic kinase activity (14). One of the earliest detectable events after TCR triggering is the tyrosine phosphorylation of the CD3/ζ chains (15, 16) on antigen recognition activation motifs (ARAMs) that are present within the cytoplasmic domain of the CD3/ζ chains (17-19). p56^{lck} or p59^{fyn} is thought to be the kinase responsible for this early phosphorylation event (20, 21). This in turn should lead to the recruitment of the tyrosine kinase ZAP-70, most likely by an interaction medi-

ated by src homology (SH)2 domain (7, 22, 23). A similar mechanism of recruitment has been proposed for the Syk tyrosine kinase (12), although recent studies by Couture et al. (13) have shown that Syk may be constitutively associated with TCR/CD3/ζ. TCR stimulation also leads to tyrosine phosphorylation and activation of ZAP-70 and Syk (references 7 and 13 and our own unpublished results).

In view of the dramatic increase in cellular tyrosine phosphorylation after TCR triggering, additional interactions between the src and Syk families of PTKs are likely to occur within the activated TCR complex. Cotransfection experiments in COS cells and in a human cytotoxic T lymphocyte line have demonstrated a functional cooperation of these two classes of PTKs (8, 9, 12, 21). We have recently reported that a direct physical interaction between p56^{lck} and ZAP-70 can occur during the course of T cell activation and that p56^{lck} binds, via its SH2 domain, to tyrosine-phosphorylated ZAP-70 after anti-CD3 stimulation of Jurkat cells (24). We have proposed that this interaction takes place as soon as ZAP-70 is phosphorylated on an unknown tyrosine residue that serves as an adaptor site for the SH2 domain of p56^{lck}. In addition, an SH2-mediated interaction of p56^{lck} with Syk has recently been proposed based on in vitro data (13), and an association

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[1] Abbreviations used in this paper: ARAM, antigen recognition activation motif; PTK, protein tyrosine kinase; SH, src homology.

of p56[lck] with Syk has been detected in phenylarsine oxide–treated LSTRA cells (25).

We and others (13, 24) have also suggested that an SH2-mediated interaction between p56[lck] and ZAP-70 or Syk could mediate the recruitment of p56[lck] to the activated TCR, allowing p56[lck] to interact with and/or to phosphorylate other effector molecules. The interaction of p56[lck] with CD4 or CD8 provides another clue to the possible contribution of p56[lck] to TCR signaling. It has been proposed that CD4-associated p56[lck] anchors CD4 and the stimulated TCR/CD3/ζ complex to the same MHC molecule (26). Indeed, by using fluorescence resonance energy transfer, colocalization of CD4 and the TCR/CD3/ζ complex has been shown to be dependent on the association of p56[lck] and CD4 (27). In addition, the TCR/CD4 association may be mediated by the binding of the SH2 domain of CD4-associated p56[lck] to phosphotyrosine residues within activated TCR/CD3/ζ (28). However, the target protein(s) binding to the SH2 domain of p56[lck] has not been identified in these studies.

We have taken a biochemical approach to study the potential contribution of the p56[lck] SH2 domain to the clustering of CD4 and TCR/CD3/ζ. We demonstrate that the SH2 domain of CD4-associated p56[lck] binds to two tyrosine-phosphorylated proteins, ZAP-70 and Syk, after anti-CD3 activation of Jurkat cells. We also show biochemical evidence for an SH2-dependent association of p56[lck]/CD4 with TCR/CD3/ζ. In addition, ZAP-70 is shown to colocalize with CD4 in activated T lymphoblasts. Based on these data, we propose that the interaction of the SH2 domain of CD4-associated p56[lck] with tyrosine-phosphorylated, ζ-associated ZAP-70 and/or Syk is responsible for the activation-dependent association of CD4 with the TCR/CD3/ζ complex.

Materials and Methods

Cells Lines and Antibodies. Jurkat cells, clone 77-6.8 (a gift from Dr. K. A. Smith, Dartmouth Medical School, Hanover, NH), were grown in RPMI 1640 medium supplemented with 10% FCS (RPMI-FCS), 2 mM L-glutamine, penicillin, and streptomycin. CD4⁻ and CD4⁺ clones were derived from clone 77-6.8 by fluorescence-activated cell sorting and cloning. Human T lymphoblasts were obtained by culturing PBMCs at a concentration of 10⁶ cells/ml in RPMI-FCS with 2 μg/ml PHA (Wellcome Industries, Beckenham, UK) for 5 d. After washing, the cells were expanded and maintained in RPMI-FCS supplemented with recombinant human IL-2 (50 IU/ml; kindly provided by P. Dellabona, Dipartimento di Biologia e Tecnologia, Milano, Italy).

mAbs used included CBT3 (29) (kindly provided by L. De Monte, University of Turin, Turin, Italy) and biotin-labeled Leu4 (Becton Dickinson & Co., Mountain View, CA), both specific for human CD3; anti-CD3ε (2Ad2A2, IgM) and anti-TCR Vβ8 (5Rex-9H5, IgG2a) (kindly provided by E. Reinhertz, Dana Farber Cancer Institute, Boston, MA); anti–human CD4 B66.6.1 (IgG1; a gift of B. Perussia, Jefferson Cancer Center, Philadelphia, PA), 19Thy-5D7 (IgG2a; kindly provided by E. Reinherz), and biotin-labeled Leu3a (Becton Dickinson); and antiphosphotyrosine (4G10, IgG2b; Upstate Biotechnology Inc., Lake Placid, NY).

Rabbit polyclonal antibodies used included anti–human p56[lck]

kinase directed against the COOH-terminal region (Upstate Biotechnology Inc.); anti-p56[lck] serum directed against a synthetic peptide corresponding to amino acids 39–64 of p56[lck] (the NH₂-terminal region; kindly provided by S. Fischer, Hôpital Cochin, Paris, France); anti-p56[lck] serum raised against recombinant human p56[lck] and antiphosphotyrosine serum (kindly provided by M. Gassmann and P. Burn, Hoffmann-La Roche Ltd., Basel, Switzerland); anti-ζ serum (a gift from J. Ravetch, Memorial Sloan-Kettering Institute, New York, NY); anti-Syk antiserum (K423; kindly provided by K. Robbins, National Institutes of Health, Bethesda, MD); and anti–ZAP-70 antiserum directed against a synthetic peptide corresponding to amino acids 485–499 of ZAP-70 (produced in our laboratory using KLH as a carrier as described [8]).

Immunoprecipitations. Jurkat cells were washed twice in RPMI 1640 medium and resuspended at 10⁸ cells/ml in RPMI 1640. Cells were preincubated for 10 min at 37°C and stimulated with anti-CD3 mAb (2Ad2A2 at 1:200 dilution of ascites) for the time indicated. Cells were harvested and solubilized at 10⁸ cells/ml for 30 min at 4°C in 1% Brij 96 or 1% NP-40 lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-sc [Interchim, Paris, France], 50 mM NaF, 10 mM Na₄P₂O₇, and 1 mM NaVO₄). In peptide competition experiments, 100 μM unphosphorylated or tyrosine-phosphorylated peptide was added to the lysis buffer as indicated. Postnuclear lysates were precleared for 1 h with maltose-binding protein–Sepharose beads and subjected to immunoprecipitation for 2 h with antibodies preadsorbed to protein A–Sepharose (Pharmacia Diagnostics AB, Uppsala, Sweden) or covalently coupled to protein A–Sepharose (30). Precipitation of SH2-binding proteins was done as described previously (24). Precipitates were washed twice in 1% detergent lysis buffer without inhibitors and twice with 0.05% detergent lysis buffer and boiled in sample buffer before electrophoresis.

Biotinylation of Cell Surface Proteins. Jurkat cells were washed twice in PBS (10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, and 137 mM NaCl) and resuspended at 10⁸ cells/ml in 5 mM NHS-LC Biotin (Pierce Chemical Co., Rockford, IL) in PBS. Cells were incubated for 1 h at 4°C, washed twice with 20 mM PBS-glycine, and twice in PBS, and finally resuspended in RPMI 1640 medium at 10⁸ cells/ml. Activation and immunoprecipitations were then performed as previously described.

Immunoblotting. Immunoblotting and detection of proteins were performed as described previously. For detection of biotinylated proteins, blots were blocked in PBS plus 0.1% Tween 20 in the presence of 5% BSA, incubated with a 1:3,000 dilution of streptavidin-biotinylated horseradish peroxidase complex (Amersham International, Buckinghamshire, UK), and developed with the enhanced chemiluminescence detection system (Amersham International).

In Vitro Kinase Assay and Reimmunoprecipitation. Immunocomplexes were prepared as previously described except that an additional wash of the beads with kinase buffer (25 mM Hepes, pH 7.3, 10 mM MnCl₂, 0.05% Brij 96) was performed. Reactions were started by the addition of 30 μl of kinase buffer containing 10 μCi of [γ³²P]ATP (3,000 Ci/mM), incubated at room temperature under constant agitation, and stopped after 15 min by the addition of 2× sample buffer containing 10 mM EDTA. For reimmunoprecipitations, reactions were stopped by adding 10 mM EDTA. After washing twice with 1% Brij lysis buffer, the beads were resuspended in elution buffer containing 1% SDS and 2 mM DTT in 0.05% Brij lysis buffer with inhibitors. Samples were incubated at 68°C for 5 min and then cooled on ice. After addition of 20 mM iodoacetamide,

supernatants were diluted sevenfold with 1% Brij lysis buffer with inhibitors and kept on ice for at least 1 h before reimmunoprecipitation. Samples were analyzed by SDS-PAGE. Gels were fixed, treated with 1 M KOH for 1 h at 55°C to remove the alkali-labile phosphate groups from serine- and threonine-phosphorylated proteins, dried, and autoradiographed.

Capping and Immunofluorescence Microscopy. 2-mo-old, PHA-activated human lymphoblasts at a concentration of 10^7 cells per ml in RPMI plus 5% FCS were incubated for 45 min with biotin-labeled Leu4 or Leu3a (10 μg/ml). After washing, cells were incubated with fluorescein-conjugated streptavidin (Protos Immunoresearch, San Francisco, CA) at 5 μg/ml. All these steps were performed at 4°C. After washing, the cells were either immediately plated on a poly-L-lysine–coated glass coverslip or shifted to 37°C for 5 min to induce capping and then plated. The cells were then fixed with 3% paraformaldehyde in PBS at room temperature for 7 min, permeabilized with 0.2% Tween 20 in PBS for 5 min, and immunolabeled with affinity-purified rabbit antiphosphotyrosine, anti-p56^{kk}, or anti–ZAP-70 antisera, followed by rhodamine-conjugated F(ab')$_2$ fragments of goat anti–rabbit IgG (Protos Immunoresearch) at 5 μg/ml. In the case of CD4 capping, some of the cells were previously incubated with CBT3 mAb, washed, and shifted to 37°C for 1 min to induce CD3-mediated cell activation.

Results

Tyrosine-Phosphorylated Syk and ZAP-70 Bind to p56^{lck} during T Cell Activation. We have previously shown that after CD3 stimulation of Jurkat cells, p56^{lck} associates with two tyrosine-phosphorylated proteins, the tyrosine kinase ZAP-70 and another unidentified protein of ~74 kD (24). In addition, in an in vitro kinase assay, we detected ZAP-70 and another tyrosine-phosphorylated protein of 72–74 kD that binds to the SH2 domain of p56^{lck} after CD3 cross-linking, suggesting that the latter may also have tyrosine kinase activity. The protein tyrosine kinase Syk, a 72-kD homologue of ZAP-70, has been shown to be well expressed in Jurkat cells (12, 31) and therefore represents a likely candidate for

the second unidentified protein that coimmunoprecipitates with p56^{kk}.

To test this hypothesis, we performed immunoprecipitations of cellular extracts of activated and unstimulated Jurkat T cells with anti-Syk, anti–ZAP-70, and anti-p56^{kk} antibodies followed by antiphosphotyrosine immunoblotting (Fig. 1 *A*). In anti-Syk immunoprecipitates, Syk was detected as a tyrosine-phosphorylated band of 74 kD. Its tyrosine phosphorylation strongly increased after Jurkat cell activation (the high basal level of tyrosine phosphorylation detected in this experiment was not consistently detected; see also Fig. 3, lanes *3* and *4*). Similarly, ZAP-70, migrating as a 68/70-kD doublet, became heavily tyrosine phosphorylated after CD3 cross-linking, whereas only a small fraction of tyrosine-phosphorylated ZAP-70 was detected in unstimulated cells after longer exposures (data not shown). ZAP-70 and Syk comigrated with tyrosine-phosphorylated proteins coprecipitating with p56^{lck} after cell activation (Fig. 1 *A*). Their amounts were too low to be detectable by Western blotting using our anti–ZAP-70 or anti-Syk antibodies. To identify both proteins formally, we performed reimmunoprecipitations of ZAP-70 and Syk from p56^{lck} immunoprecipitates subjected to an in vitro kinase assay (Fig. 1 *B*). In this assay p56^{lck} became strongly labeled; in addition, a broad band of 70–74 kD became labeled in the anti-p56^{kk} precipitate after CD3 cross-linking. Reprecipitation experiments from this sample with specific anti–ZAP-70 and anti-Syk antisera clearly demonstrated that ZAP-70 and Syk form part of the 70–74-kD band (Fig. 1 *B*). Furthermore, the identification of the two kinases was confirmed by immunodepletion and antiphosphotyrosine Western blot analysis of anti-p56^{kk} immunoprecipitates from activated cells (Fig. 2). After immunodepletion of ZAP-70 (Fig. 2 *A*, lanes *3* and *4*), the 68/70-kD doublet was no longer detectable in p56^{kk} immunoprecipitates (lanes *1* and *2*). Conversely, the 74-kD band coprecipitating with p56^{kk} disappeared after immunodepletion of Syk (Fig. 2 *B*). Importantly,
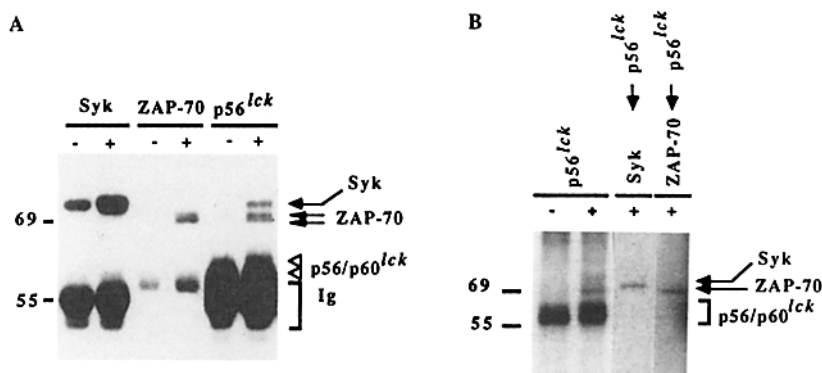

Figure 1. Syk and ZAP-70 bind to p56^{kk} after T cell activation. (*A*) Brij 96 lysates were prepared from unstimulated Jurkat cells (−) or from cells stimulated with anti-CD3 for 2 min (+). Immunoprecipitations were performed using 10^7 cell equivalent with anti-Syk and anti-p56^{kk} antibodies or 2.5 × 10^6 cell equivalent with anti–ZAP-70 antiserum. Samples were loaded on an 8% SDS–polyacrylamide gel and analyzed by antiphosphotyrosine immunoblotting. The relative positions of Syk and ZAP-70 (migrating as a doublet) are shown by arrows. The p56^{kk} protein is indicated by open arrowheads. A longer exposure (3 min instead of 20 s) is shown for anti-p56^{kk} immunoprecipitates. Positions of molecular mass markers are shown in kilodaltons. (*B*) Brij 96 lysate was prepared from 3 × 10^7 Jurkat cells before (−) or after (+) stimulation with anti-CD3 for 2 min and subjected to immunoprecipitation with anti-p56^{kk}. Washed immunoprecipitates were incubated in an in vitro kinase assay with [γ-^{32}P]ATP. One-third of the samples were directly eluted by boiling in sample buffer (p56^{kk}), and (in the case of the sample from stimulated cells) the remaining two-thirds were subjected to reimmunoprecipitation by anti–ZAP-70 or anti-Syk antibodies (p56^{kk} → ZAP-70 and p56^{kk} → Syk), respectively. Phosphorylated proteins were analyzed by 12% SDS-PAGE, KOH treated, and autoradiographed. A longer exposure (6 d instead of 1 d) is shown for reimmunoprecipitates. The relative positions of p56^{kk}, ZAP-70, and Syk are indicated.
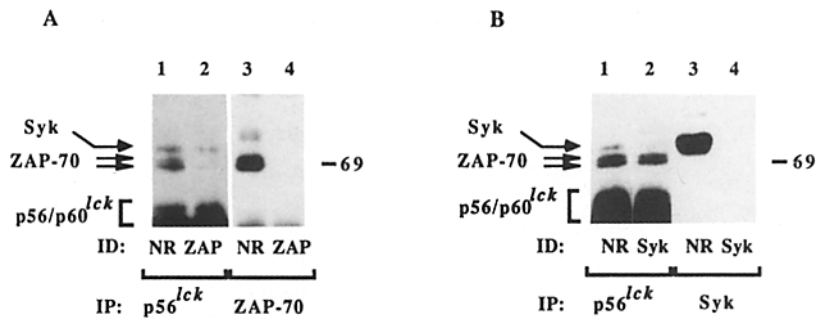
immunodepletion of ZAP-70 did not appear to affect the p56[lck]/Syk interaction (Fig. 2 A, lanes 1 and 2), nor did immunodepletion of Syk affect the p56[lck]/ZAP-70 interaction (Fig. 2 B, lanes 1 and 2), suggesting that Syk and ZAP-70 bind to independent populations of p56[lck].

Interaction of Tyrosine-Phosphorylated Syk and ZAP-70 with the p56[lck] SH2 Domain. After Jurkat activation, tyrosine-phosphorylated ZAP-70 binds to the SH2 domain of p56[lck] in vitro (24). To test whether a similar type of interaction occurs between p56[lck] and Syk, we analyzed SH2-binding proteins and anti-Syk immunoprecipitates by antiphosphotyrosine and anti-Syk immunoblotting (Fig. 3). In unstimulated Jurkat cells, Syk migrated as a 72-kD band (Fig. 3, lane 7). After cell activation, an additional more slowly migrating form of Syk could be detected by anti-Syk immunoblotting (Fig. 3, lane 8). Only this 74-kD species, which corresponds to the tyrosine-phosphorylated form of Syk (Fig. 3, lane 4), bound to the SH2 domain of p56[lck] after activation (lanes 1, 2, 5, and 6).

To investigate whether the in vivo association of ZAP-70 and Syk with p56[lck] is an SH2-mediated interaction, cellular
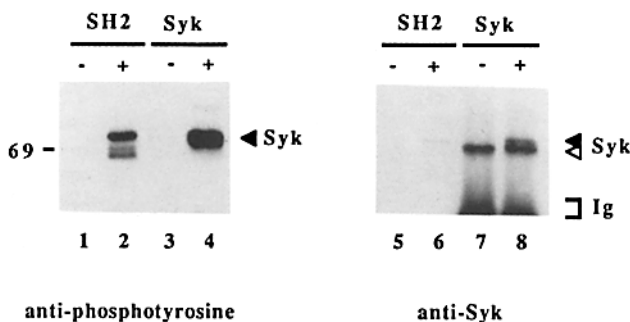

Figure 3. Tyrosine-phosphorylated Syk binds to the SH2 domain of p56[lck] in vitro. NP-40 lysates were prepared from 10[7] unstimulated Jurkat cells (−) or 10[7] cells stimulated with anti-CD3 for 2 min (+). Proteins associated with Sepharose-coupled maltose-binding protein SH2, and anti-Syk immunoprecipitates were separated on an 8% SDS-polyacrylamide gel and analyzed by antiphosphotyrosine or anti-Syk immunoblotting. The positions of unphosphorylated and tyrosine-phosphorylated Syk are indicated by open and closed arrowheads, respectively.

extracts from unstimulated or activated Jurkat cells were lysed in the presence of an unphosphorylated or tyrosine-phosphorylated peptide corresponding to a sequence (YEEI) predicted to be optimal for binding to the src family kinases (32). Extracts were analyzed for the coprecipitation of ZAP-70 and Syk with p56[lck] using two different antibodies directed against the NH2 and COOH termini of the p56[lck] protein (Fig. 4 A). Using either antibody, tyrosine-phosphorylated ZAP-70 and Syk were detected in anti-p56[lck] precipitates of activated cells (Fig. 4 A, lanes 2 and 5). The phosphorylated but not the unphosphorylated version of the p56[lck] SH2 competitor peptide was able to impede the association between p56[lck] and the Syk/ZAP family kinases (Fig. 4 A, compare lanes 3 and 6 with lanes 2 and 5, respectively). Under the same conditions, the phosphorylated peptide competed efficiently for the binding of tyrosine-phosphorylated proteins to the recombinant SH2 domain of p56[lck] (Fig. 4 A, lanes 7 and 8). We therefore conclude that in CD3-stimulated cells, tyrosine-phosphorylated Syk and ZAP-70 bind to p56[lck] by an SH2-mediated interaction.

Syk- and ZAP-70-mediated Interaction of p56[lck] with the Activated TCR/CD3/ζ Complex. ZAP-70 as well as Syk is recruited to the TCR by association with tyrosine-phosphorylated CD3 and ζ chains (references 7, 12, 22, and 33 and our own unpublished results). Indeed, using a sensitive in vitro kinase assay, the presence of Syk and ZAP-70 together with CD3/ζ chains could be detected in anti-p56[lck] immunoprecipitates of activated Jurkat cells (Fig. 4 B, lanes 1 and 2). This detection was abolished in the presence of a tyrosine-phosphorylated peptide containing the sequence YEEI (Fig. 4 B, compare lanes 2 and 3). Conversely, the association of radioactively labeled p56[lck] with anti-ζ immunoprecipitates of activated cells was significantly reduced in the presence of the competitor peptide, whereas the detection of ζ-associated ZAP-70 and Syk was essentially unaffected (Fig. 4 B, lanes 4–6), possibly because this association is mediated by the binding of two SH2 domains to a doubly tyrosine-phosphorylated ARAM on the ζ chain (21, 23). The slightly lower degree of CD3/ζ phosphorylation in the in vitro kinase assay seen in the presence of the competitor peptide (Fig. 4 B, compare lanes 5 and 6) most probably
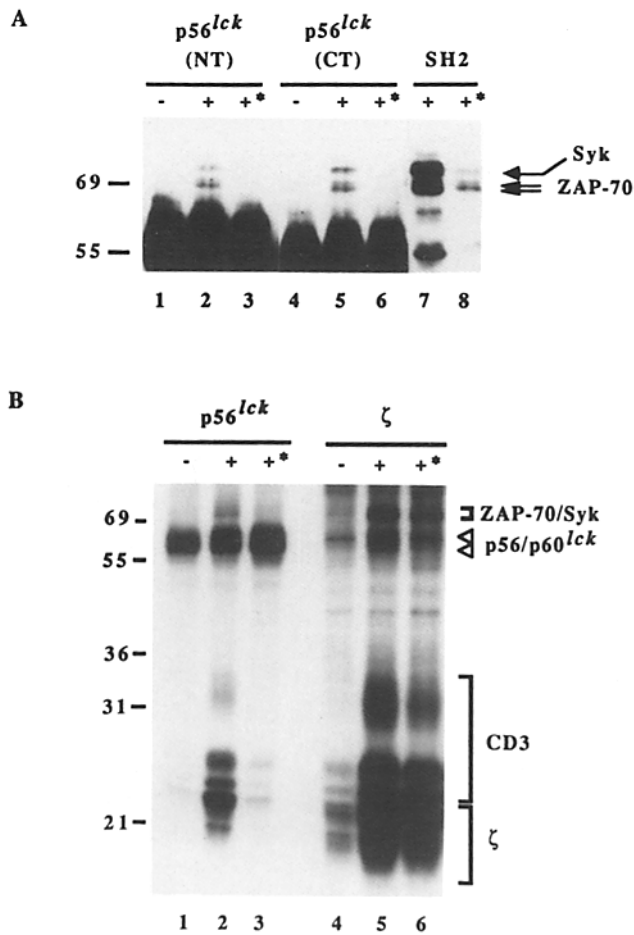
A

B

Figure 4. Interaction of tyrosine-phosphorylated Syk and ZAP-70 with the SH2 domain of p56^{lck}. (A) 10^7 unstimulated Jurkat cells (−) or 10^7 cells stimulated with anti-CD3 for 2 min (+) were lysed in Brij 96 lysis buffer containing 100 μM unphosphorylated or tyrosine-phosphorylated (*) peptide EPQYEEIPI. Proteins associated with Sepharose-coupled MBP SH2 and anti-p56^{lck} immunoprecipitates performed with antisera directed against the NH$_2$- (NT) or COOH- (CT) terminal regions of the p56^{lck} protein were separated by 8% SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting. Positions of molecular mass markers are shown in kilodaltons. (B) 10^7 unstimulated Jurkat cells (−) or 10^7 cells stimulated with anti-CD3 for 2 min (+) were lysed in Brij 96 lysis buffer containing 100 μM unphosphorylated or tyrosine-phosphorylated (*) peptide EPQYEEIPI. Anti-p56^{lck} (to the COOH-terminal region) and anti-ζ immunoprecipitates were incubated in an in vitro kinase assay with [γ-^{32}P]ATP, and phosphorylated proteins were analyzed by 12% SDS-PAGE, KOH treatment, and autoradiography. The positions of p56^{lck}, ZAP-70/Syk (distinguishable by reimmunoprecipitation as shown in Fig. 1 B, but not resolved on a 12% gel), the CD3, and ζ chains are indicated.

results from the reduced level of associated p56^{lck}, which may contribute to the overall phosphorylation of CD3/ζ in the assay. The tyrosine-phosphorylated peptide did not affect the levels of phosphorylated ζ and CD3 chains detected in anti–ZAP-70 and anti-Syk immunoprecipitates by antiphosphotyrosine blotting (data not shown). Thus, the tyrosine-phosphorylated peptide specifically competes for the p56^{lck}/ZAP-70 and p56^{lck}/Syk interaction. Although we cannot formally exclude the possibility of a direct p56^{lck}/ζ association, our previous findings (24) demonstrated that the p56^{lck}

SH2 domain binds directly to tyrosine-phosphorylated ZAP-70 (24) and Syk (data not shown), and they argued against tyrosine-phosphorylated CD3/ζ binding directly to the p56^{lck} SH2 domain (see Discussion).

Together, these results suggest an activation-dependent association of p56^{lck} with ζ/ZAP-70 and ζ/Syk, most likely mediated by the binding of tyrosine-phosphorylated ZAP-70 or Syk to the SH2 domain of p56^{lck}.

SH2-mediated Interaction of p56^{lck}/CD4 with the Activated TCR Complex. It is well established that a large fraction of p56^{lck} binds to CD4 (reviewed by Rudd et al. [34]). To test whether CD4-associated p56^{lck} is involved in the described SH2-mediated interaction with ZAP-70 and Syk, we analyzed anti-CD4 immunoprecipitates for the presence of ZAP-70 and Syk by antiphosphotyrosine immunoblotting (Fig. 5). Indeed, CD4-associated p56^{lck} interacts with tyrosine-phosphorylated Syk and ZAP-70 after activation (Fig. 5, compare both + lanes).

It has been proposed that the binding of the SH2 domain of CD4-associated p56^{lck} to tyrosine-phosphorylated proteins within the activated TCR complex may lead to the assembly of a signaling complex (28). One prediction of this model would be an activation-dependent association of CD4 with the TCR. We have studied the influence of T cell activation on TCR/CD4 association by biotinylation of cell surface proteins. After cell surface labeling, cells were kept unstimulated or activated with an anti-CD3 antibody. Immunoprecipitates of CD4 and the TCR β chain from lysates of surface-labeled cells were subjected to SDS-PAGE under reducing and nonreducing conditions and analyzed by Western blotting with streptavidin–biotinylated horseradish peroxidase complex (Fig. 6 A). Using this technique, CD4 and the TCR α and β chains were clearly detected in anti-CD4 and anti-TCR Vβ precipitates, respectively (Fig. 6 A, lanes 3–9). In addition, an activation-dependent coprecipitate of a fraction of CD4 with the TCR was detected in CD4$^+$ but not in the corresponding CD4$^-$ Jurkat cells (Fig. 6 A, compare lanes 3 and 4 with lanes 1 and 2). We also detected an activation-dependent association of a small fraction of a 63–67-kD band, likely to correspond to CD5 (references 35 and 36 and data not shown), with the TCR in CD4$^+$ or CD4$^-$ Jurkat cells (Fig. 6 A, lanes 1–4). The CD4$^+$ and CD4$^-$ Jurkat clones used for this experiment expressed at their cell surface comparable amounts of CD3, and CD3–cross-linking led to indistinguishable early (i.e., increase in intracellular Ca^{2+}, tyrosine phos-

Figure 5. Syk and ZAP-70 associate with p56^{lck}/CD4. 10^7 unstimulated Jurkat cells (−) or 10^7 cells stimulated with anti-CD3 for 1.5 min (+) were lysed in Brij 96 lysis buffer. Anti-p56^{lck} (to the COOH-terminal region) and anti-CD4 (B66.6.1) immunoprecipitates were separated by 8% SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting. Arrows indicate the positions of ZAP-70 and Syk.
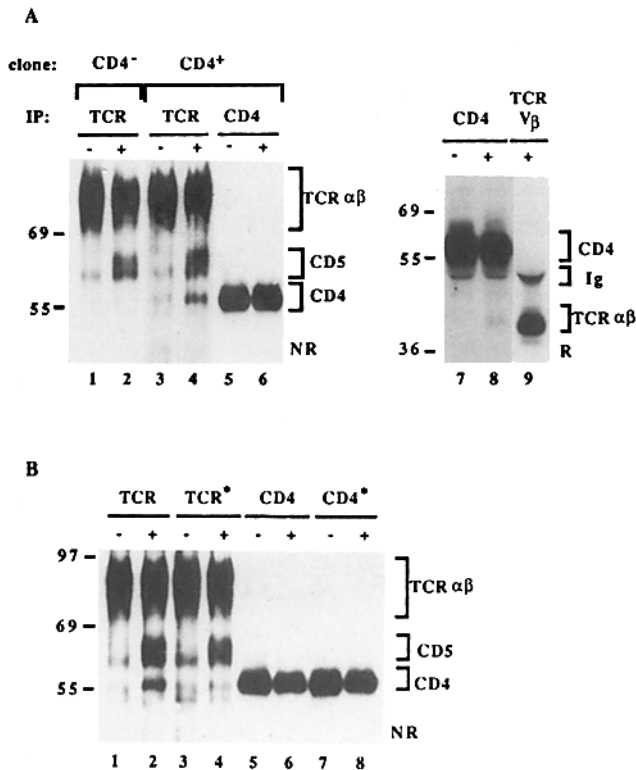
A

B

Figure 6. SH2-mediated interaction of p56^{lck}/CD4 with the activated TCR complex. (*A*) CD4$^+$ or CD4$^-$ Jurkat cells were surface biotinylated, and Brij 96 lysates were prepared from 10^7 Jurkat cells before (−) or after (+) stimulation with anti-CD3 for 1.5 min. Immunoprecipitates with antibodies directed against CD4 (19Thy-5D7) and TCR Vβ (9H5) were separated by 7% SDS-PAGE under nonreducing (*NR*) or by 10% SDS-PAGE under reducing (*R*) conditions, transferred to nitrocellulose, and revealed with streptavidin–biotinylated horseradish peroxidase complex. A longer exposure is shown for lanes *1–4* (3 min) compared with lanes *5* and *6* (20 s) and for lanes *7* and *8* (2 min) compared with lane *9* (10 s). The positions of CD5 (determined by comparison with an anti-CD5 immunoprecipitation; data not shown), CD4, and the TCR αβ chains are indicated. Positions of molecular mass markers are shown in kilodaltons. (*B*) Same as in *A*, but lysates were prepared in Brij 96 lysis buffer containing 500 μM unphosphorylated or tyrosine-phosphorylated (*) peptide EP-QEEIPI. Samples were analyzed on a 7% SDS gel under nonreducing (*NR*) conditions. Exposition times were identical for all lanes, but only 30% of the sample was loaded for anti-CD4 immunoprecipitates.

phorylation of cellular proteins) and late (i.e., IL-2 secretion) events of activation (data not shown). The activation-dependent association of CD4 with the TCR was confirmed by the presence of a small proportion of TCR α/β chains coprecipitating with CD4 from activated cells (Fig. 6 *A*, lanes 7–9; not visible in lanes 5 and 6 because only 30% of the samples were loaded).

As has been shown, a tyrosine-phosphorylated peptide containing the sequence YEEI efficiently competed for the p56^{lck}/ZAP-70 or p56^{lck}/Syk interaction without affecting the ζ/ZAP-70 or ζ/Syk interaction (Fig. 4 and data not shown). Fig. 6 *B* shows that the same tyrosine-phosphorylated peptide, but not the corresponding unphosphorylated version, was able to outcompete the activation-dependent association of CD4 with the TCR (Fig. 6 *B*, compare lanes

2 and 4), but not the low basal level of association in unstimulated cells (compare lanes 1, 3, and 4). Moreover, a shorter exposure of the blot shows that no change was detected in the total CD4 present in the cell lysate before or after activation in the presence of the phosphorylated peptide (Fig. 6 *B*, lanes 5–8). Note that the association of the TCR with the 63–67-kD band likely to correspond to CD5 did not appear to be affected by the tyrosine-phosphorylated peptide (Fig. 6 *B*, lanes 1–4); therefore, this association is unlikely to be mediated by the same mechanism as the TCR/CD4 association.

Together, these results suggest that upon activation, p56^{lck}/CD4 associates with the activated TCR/CD3/ζ complex by an SH2-mediated interaction, most likely involving the binding of p56^{lck} SH2 to tyrosine-phosphorylated ZAP-70 or Syk.

ZAP-70 Cocaps with CD4 in Activated T Cells. To provide evidence in intact cells for the occurrence of the activation-mediated associations of signaling molecules described here, we examined the intracellular redistribution of p56^{lck} and ZAP-70 after antibody-mediated CD3 and CD4 capping. Because of the rapid internalization of CD3 caps and the high level of p56^{lck} in Jurkat cells (data not shown), a human PBL-
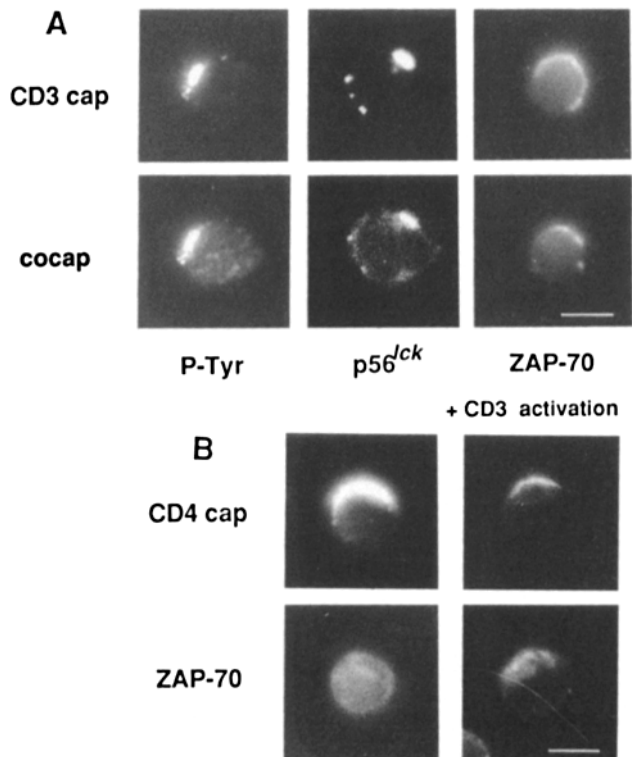

Figure 7. ZAP-70 cocaps with CD4 in activated T cells. Antibody-mediated CD3 and CD4 capping was induced on the T cell surface, and cells were counterstained for phosphotyrosine, p56^{lck}, and ZAP-70. (*Upper panels*) Double indirect immunofluorescence labeling of cell surface CD3 (green channel) and intracellular phosphotyrosine, p56^{lck}, and ZAP-70 (red channel). (*Lower panels*) Double indirect immunofluorescence labeling of cell surface CD4 (green channel) and intracellular ZAP-70 (red channel). Each cell is represented by two photographs in a column. Bar, 5 μm.

derived T cell line was chosen for this analysis. As shown by double immunofluorescence in Fig. 7 (*upper panels*), CD3 capping resulted in colocalization of tyrosine-phosphorylated proteins. Similarly, p56^{lck} and ZAP-70 were in part migrating in the cap region. The same results were obtained with capping using anti-TCRαβ mAb (data not shown).

If the association of CD4 with the TCR is mediated by an activation-dependent p56^{lck}/ZAP-70 interaction, ZAP-70 should be found in close proximity to CD4 after CD3 stimulation. To detect such a potential association in vivo, CD4 molecules on the surface of unstimulated or activated human T lymphocytes were collected into caps, and the subcellular distribution of ZAP-70 was analyzed (Fig. 7, *lower panels*). In resting cells, no redistribution of ZAP-70 was observed, whereas after 1 min of stimulation of the cells with anti-CD3 antibodies, a clear enrichment of ZAP-70 under the CD4 cap was observed. These results demonstrate the requirement of CD3 stimulation for ZAP-70/CD4 cocapping.

Discussion

T cell activation requires the recognition of MHC-presented peptide antigen by the TCR. During this process, CD4 and the TCR are thought to interact with the same MHC II molecule in a stable ternary complex (reviewed by Janeway [26]). It has been demonstrated for T cell clones that cellular activation is required to induce redistribution of CD4 into the proximity of the TCR, as shown by cocapping, comodulation, and fluorescence resonance energy transfer (37–39). However, no direct biochemical evidence for an activation-dependent association of CD4 with the TCR/CD3/ζ has been reported.

We have shown, using cell surface biotinylation, that in Jurkat cells, CD4 associates with TCR/CD3/ζ after CD3 triggering. What is the driving force for this activation-dependent colocalization? Several observations point to a central role of CD4-associated p56^{lck} in the formation of the multimolecular TCR/CD3/ζ–CD4 complex. Collins et al. (27) have shown that the interaction of p56^{lck} with CD4 is critical for the colocalization of CD4 with TCR/CD3/ζ and for signal transduction. Furthermore, coreceptor function of p56^{lck}/CD4 does not seem to depend on the active kinase domain of p56^{lck}, but rather on its SH2 or SH3 domain (28, 40). Therefore, one of the functions of p56^{lck} may be to link CD4 physically to the TCR via its SH2 or SH3 domain.

Our data are consistent with the idea that during T cell activation, CD4 associates with TCR/CD3/ζ via the SH2 domain of p56^{lck} and that ZAP-70 and/or Syk represent the molecular link between them. First, we show that a tyrosine-phosphorylated peptide which binds with high affinity to the SH2 domain of p56^{lck} is able to disrupt the activation-dependent association of CD4 with TCR/CD3/ζ. Second, after activation, ZAP-70 and Syk can be coimmunoprecipitated with CD4. Third, we show that under the same conditions, p56^{lck} binds to ZAP-70 and Syk and that this interaction can be prevented by the presence of the same tyrosine-phosphorylated peptide; moreover, binding of ZAP-70 (24) and Syk

to a recombinant SH2 domain of p56^{lck} was demonstrated. Fourth, by in vitro kinase assay, we have detected the association of ζ with p56^{lck}/Syk and p56^{lck}/ZAP-70 and, vice versa, the association of p56^{lck} with ζ in anti-ζ immunoprecipitates in the course of CD3 stimulation of Jurkat cells. The tyrosine-phosphorylated peptide was able to compete specifically for the p56^{lck}/ZAP-70 and p56^{lck}/Syk interactions without affecting the ζ/ZAP-70 and ζ/Syk interactions. Finally, experiments in intact cells using T lymphoblasts provided additional support to these findings. p56^{lck} was shown to cocap with CD3 rapidly upon activation, which is in agreement with recent data from Ley et al. (41), who also found p56^{lyn} not to be localized at the plasma membrane. Also, in separate experiments, we observed that ZAP-70 colocalizes with CD3 or CD4 only after activation. Although the immunofluorescence experiments provide correlative evidences, together they are consistent with an activation-induced redistribution of p56^{lck}/CD4 in the vicinity of ZAP-70 bound to the TCR/CD3/ζ complex.

In accordance with our results, it has been shown that the expression of ζ in the TCR/CD3 complex is of critical importance for p56^{lck}/CD4-mediated up-regulation of TCR/CD3 signaling in the human leukemic T cell line HBP-ALL (42). Moreover, Dianziani et al. (43) have reported that tyrosine phosphorylation of the ζ chain parallels the physical association of CD4 and the TCR in a murine T cell clone.

Our previous findings (24) demonstrated that the p56^{lck} SH2 domain binds directly to tyrosine-phosphorylated ZAP-70 and to a p74 tyrosine–phosphorylated protein (identified here as being Syk) and argued against a direct binding to tyrosine-phosphorylated CD3/ζ chains. Using the p56^{lck} SH2 domain as a probe in far Western blot experiments, tyrosine-phosphorylated ZAP-70 (24) and Syk (data not shown) could be directly recognized, whereas CD3/ζ was barely detectable (24). Moreover, in cell lysates from activated Jurkat cells, the p56^{lck} SH2 domain readily precipitated tyrosine-phosphorylated ZAP-70 and p74 (Syk), whereas only little tyrosine-phosphorylated CD3/ζ was detectable, even though it was abundantly present in anti-ζ immunoprecipitates (24). The most likely interpretation of this result is that the small amount of tyrosine-phosphorylated CD3/ζ observed with the p56^{lck} SH2 domain corresponds to the fraction bound to ZAP-70 and Syk. Indeed, the ratio of tyrosine-phosphorylated ZAP-70 to CD3/ζ in anti–ZAP-70 immunoprecipitates is similar to that detected with the p56^{lck} SH2 domain (24). In addition, we have shown that the presence of CD3/ζ is not required for the p56^{lck} SH2–mediated binding of ZAP-70 and Syk, since depletion of CD3/ζ from a lysate of activated cells does not prevent binding of the remaining ZAP-70/Syk to the p56^{lck} SH2 domain (24). In accordance with our results, Wange et al. (23) have recently demonstrated the preferential binding of ζ and CD3ε chains by ZAP-70 (SH2)$_2$ domains, whereas the p56^{lck} SH2 domain preferentially binds to a p70 phosphoprotein. Moreover, Iwashima et al. (21), using activated Jurkat cells, have demonstrated binding of ZAP-70, but not of p56^{lck}, to a doubly tyrosine-phosphorylated peptide encompassing a ζ ARAM. Together, these results argue against a direct SH2-mediated binding of p56^{lck} to CD3/ζ.
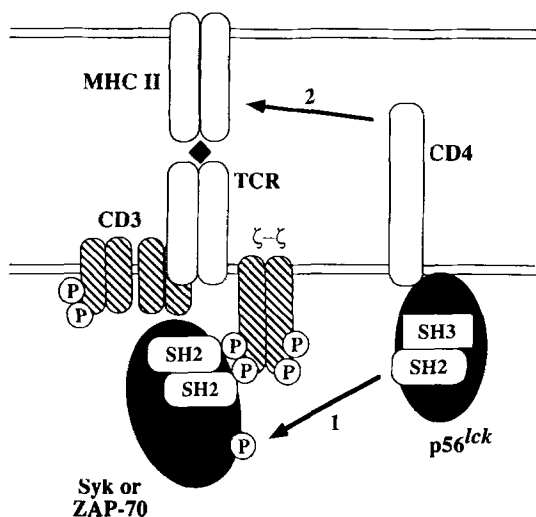
Figure 8. Hypothetical model of the recruitment of CD4 to the TCR/CD3/ζ complex. T cell receptor stimulation by MHC-bound antigen leads to tyrosine phosphorylation of the CD3/ζ chains and recruitment and tyrosine phosphorylation of the Syk or ZAP-70 tyrosine kinase. Binding of the SH2 domain of p56^lck to a phosphotyrosine residue on ZAP-70 or Syk (1) may then recruit CD4 to the activated TCR/CD3/ζ complex (2) and stabilize the interaction with the antigen-presenting MHC II molecule.

In view of our results and based on a previous model by Xu and Littman (28), we propose that the interaction of the SH2 domain of p56^lck with CD3/ζ-associated ZAP-70 and Syk enables the TCR/CD3/ζ complex to associate with the CD4 coreceptor (Fig. 8). This implies that a primary encounter of the TCR with the antigen/MHC II should lead to a partial activation (i.e., tyrosine phosphorylation of CD3/ζ chains and binding of ZAP-70 and/or Syk) of the TCR complex, which may then recruit p56^lck/CD4 with the consequence of (a) a physical stabilization, by CD4, of the TCR/antigen/MHC interaction and (b) an increased tyrosine phosphorylation of TCR-associated signaling molecules by p56^lck, thereby further amplifying the signaling process.

An interesting consequence of such a model is that CD4 would be preferentially recruited by the rare TCRs successfully engaged with the small number of specific ligands available on an antigen-presenting cell (44), resulting in an efficient recognition/signal transduction process.

A direct physical interaction between CD4 and TCR/CD3/ζ has been reported to occur independently of activation in T cell clones (45, 46), in tumor cell lines (47), and in freshly isolated T cells (48, 49). We have also observed a low level of association of CD4 with the TCR in Brij 96 lysates of unstimulated Jurkat cells, but in contrast to the association seen for activated cells, the former does not seem to be mediated by the same p56^lck SH2-mediated interaction, since it was not disrupted in the presence of a tyrosine-phosphorylated competitor peptide.

Our data indicate that, in Jurkat cells, both tyrosine-phosphorylated ZAP-70 and Syk bind to the SH2 domain of p56^lck and that one consequence of this interaction is the association of p56^lck/CD4 with activated TCR/CD3/ζ. This is probably not surprising considering the structural homology of these two PTKs (50). What are the relative contributions of ZAP-70 and Syk to this process? We show, by immunodepletion experiments, that ZAP-70 and Syk bind to separate cellular pools of p56^lck. This suggests that both kinases can contribute independently to the activation-dependent association of p56^lck/CD4 with the TCR/CD3 complex. Different affinities of the SH2 domain of p56^lck for tyrosine-phosphorylated Syk and ZAP-70 and differences in their level of expression, tyrosine phosphorylation, and CD3/ζ association may influence their respective importance for the clustering of CD4 and TCR/CD3 in distinct T cell subsets or in T cells of differing developmental stages. Indeed, several lines of evidence indicate that the functions of ZAP-70 and Syk are not redundant. First, the expression of ZAP-70 and Syk is developmentally regulated: Syk is expressed at higher levels in the thymus and lower levels in peripheral T cells and ZAP-70 is expressed at roughly equivalent levels in thymus and periphery (12). Furthermore, ZAP-70–deficient SCID patients lack peripheral CD8+ T cells and have normal numbers of circulating CD4+ T cells that are deficient in TCR-mediated signaling (10, 11, 51). These results suggest that Syk-mediated signaling may play a dominant role for thymic maturation of CD4+ T cells, whereas ZAP-70 is essential for signaling in mature CD4+ T cells.

Although further studies of signaling events in T cells of different functional or developmental stages are necessary to elucidate the individual roles of the Syk/ZAP-70 kinases, our data indicate that the mechanism of their coupling to the TCR and CD4 is essentially identical.

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