

The CD3 Chains of the T Cell Antigen Receptor Associate with the ZAP-70 Tyrosine Kinase and Are Tyrosine Phosphorylated after Receptor Stimulation

By David B. Straus* and Arthur Weiss*†

From the *Departments of Medicine and of Microbiology and Immunology and the †Howard Hughes Medical Institute, University of California-San Francisco, San Francisco, California 94143

Summary

Recent work indicates that signaling events resulting from stimulation of the T cell antigen receptor (TCR) can be initiated by the CD3 complex (γ , δ , ϵ) as well as the ζ chains of the receptor. To help characterize the signaling function of CD3 we examined its associated tyrosine kinase activity since induction of tyrosine phosphorylation is one of the earliest signaling events. Our results indicate that at least two kinases, lck and ZAP-70, contribute to the CD3-associated kinase activity. A likely target of this activity is the CD3 complex itself since we observed that TCR stimulation resulted in rapid tyrosine phosphorylation of the CD3 ϵ and δ chains. To examine the function of the CD3 ϵ chain in particular, we constructed a chimera that fused the extracellular and transmembrane domains of CD8 to the cytoplasmic domain of CD3 ϵ . This chimera demonstrated that CD3 ϵ was independently capable of associating with proteins having tyrosine kinase activity, including ZAP-70. Our results show that the kinase activity that associates with the CD3 complex has characteristics that are quite similar to the previously characterized ζ -associated kinase activity. This finding suggests that both these components of the TCR initiate signaling events using a common mechanism. However, differences in their signaling function could result from recognition of distinct substrates.

The T cell antigen receptor (TCR) is responsible for the recognition of antigen bound to major histocompatibility complex molecules on the surface of antigen presenting cells or target cells (1, 2). The TCR is composed of at least six distinct polypeptides. The polymorphic α and β subunits determine the antigen specificity of the receptor and are associated with the CD3 polypeptides (γ , δ , and ϵ chains), as well as a ζ dimer. The noncovalent association of the CD3 and ζ chains with the α and β polypeptides is required for efficient surface expression of the TCR. Ligation of the TCR initiates a signaling cascade which results in T cell proliferation and activation of effector functions. The initial molecular events that follow receptor stimulation include a rapid increase in the tyrosine phosphorylation of a number of proteins and the activation of the phosphatidylinositol signaling pathway. Recent work indicates that both the ζ and CD3 subunits are involved in initiating signal transduction events after receptor stimulation.

Several lines of evidence implicate the ζ subunit in TCR signal transduction. A T cell line was identified that fails to express ζ and is defective in signaling through the TCR (3). Furthermore, chimeras that fuse the cytoplasmic domain of ζ to other transmembrane proteins are expressed independently of the TCR and can initiate signaling events when stimulated with antibodies (4, 5). The ζ cytoplasmic domain

contains a short amino acid sequence, YXXL-X(6-8)-YXXL, which is repeated three times and found as a single copy in other putative signal transducing proteins including each of the CD3 chains (6). This motif alone is sufficient to initiate signaling events when expressed in a chimeric receptor that is crosslinked with antibody (7, 8).

In addition to ζ the CD3 chains also appear to have signaling function. The TCR expressed on the surface of cells that lack functional ζ chain can still transduce signals that result in the production of IL-2 (9). Like ζ , the independent signaling function of one of the CD3 chains, ϵ , was identified using a chimeric molecule to express ϵ independent of the other TCR subunits (10). Antibody crosslinking of this chimera was able to induce tyrosine phosphorylation and IL-2 production. Further analysis showed that like ζ the signaling function of ϵ was localized to the shared amino acid sequence. However, differences in the pattern of tyrosine phosphoproteins induced by crosslinking of the ϵ chimera and a ζ chimera have led to the hypothesis that ζ and CD3 couple to distinct signaling pathways.

Induction of tyrosine phosphorylation is believed to be the initial signaling event following TCR stimulation (11). Although it is not clear which tyrosine kinases are involved in the initial signaling, biochemical and genetic evidence implicates two src-family kinases: fyn and lck (12-17). In addi-

tion, recent work has identified a new tyrosine kinase, ZAP-70, that may play a central role (18, 19). This kinase is found associated with the tyrosine phosphorylated form of the ζ chain immediately after receptor stimulation. In addition, the signaling function of truncated forms of ζ correlates with the ability to associate with ZAP-70 and chimeras containing only the signal transduction motif also associate with this kinase (8).

To investigate the signaling mechanism of the CD3 component of the TCR we have examined its associated kinase activity. Our results indicate that this activity involves both the ZAP-70 and lck tyrosine kinases. A likely substrate of this kinase activity is the CD3 chains themselves since we observed that they became tyrosine phosphorylated after TCR stimulation. These characteristics suggest that CD3 initiates signaling events by a mechanism that is very similar to that used by ζ .

Materials and Methods

Cell Lines. Jurkat and JCaM1 (20) were grown in RPMI 1640 with 10% FCS, penicillin, streptomycin, and glutamine. Jurkat cells were transfected with the CD8 ϵ chimera by electroporation and clones were selected by growth in media with hygromycin. Clones were screened for surface expression of the chimera by staining with anti-CD8 antibody and fluorescein conjugated goat anti-mouse antibody and analyzed by flow cytometry. Jurkat cells transfected with CD8 ζ T22 (8) were grown in media with G418 to maintain expression of the chimera.

CD8 ϵ Chimera. A chimera between CD8 and CD8 ϵ was constructed using the polymerase chain reaction to join sequences from the CD3 ϵ cytoplasmic domain and the CD8 extracellular and transmembrane domains. The cytoplasmic domain of ϵ was amplified from Jurkat cDNA using primers that allowed hybridization to the CD8 transmembrane domain. Sequencing of several clones of the CD3 ϵ cytoplasmic domain revealed that it differed significantly from the published cDNA sequence (21), but was identical to the cytoplasmic coding region reported for the genomic sequence of ϵ (22). CD8 sequences were amplified from a CD8 cDNA plasmid, hybridized to the amplified CD3 ϵ cytoplasmic domain sequence, and further amplified to create the chimera. After nucleotide sequencing confirmed the chimera structure it was subcloned into the pCEP4 expression vector (Invitrogen, San Diego, CA) and transfected into Jurkat cells by electroporation.

Antibodies. The monoclonal antibodies used for the stimulation of the TCR were C305 (23), which recognizes the Jurkat Ti β chain, and Leu4 (Becton Dickinson and Co., Mountain View, CA), A32.1, or 235 (24), which recognize extracellular components of the CD3 chains. Leu4, A32.1, the anti-CD3 δ monoclonal antibody, SP64, supplied by C. Terhorst (Harvard Medical School, Boston, MA; reference 25), and an anti-CD3 γ peptide antiserum were used to immunoprecipitate CD3 chains. The anti-peptide antiserum 387, was used to immunoprecipitate TCR ζ (26), and anti-ZAP-70 peptide antiserum was used to immunoprecipitate ZAP-70 (19). The anti-phosphotyrosine antibody 4G10, was used for immunoprecipitation and immunoblotting (27). The anti-CD8 antibody OKT8 was used to stimulate Jurkat cells transfected with CD8 ϵ , and also to immunoprecipitate the chimeras.

Immunoprecipitations, Kinase Assays, and Phosphoamino Acid Analysis. Cells at 10^6 /ml were stimulated by incubating with 2 μ g/ml purified antibody, or 1:500 dilution of ascites, at 4°C for 20 min and/or 37°C for 2 min. Cells were harvested and lysed in 1% NP-

40, 10 mM Tris, pH 7.8, 150 mM NaCl, 1 mM PMSF, 2 μ g/ml pepstatin A, 1 μ g/ml Leupeptin, 10 mM Na pyrophosphate, 0.4 mM EDTA, 0.4 mM Na orthovanadate, and 10 mM NaF. Insoluble material was removed by centrifugation at 55,000 rpm for 10 min in a TL-100 ultracentrifuge at 4°C. Lysates were precleared by incubation with fixed *Staphylococcus aureus* cells, and normal rabbit serum or purified mouse Ig coupled to protein A-Sepharose beads. Immunoprecipitates were then formed from these cleared lysates using antibodies coupled to protein A- or protein G-Sepharose beads, and washed with lysis buffer containing 0.5 M NaCl. To analyze only receptors expressed on the cell surface the stimulating antibody that remained bound to the receptors was used for the immunoprecipitation. In this case lysates were precleared only with Sepharose beads, and the antibody-receptor complexes were collected by the addition of protein A- or protein G-Sepharose beads.

For kinase assays immunoprecipitates were washed sequentially with lysis buffer, 0.5 M LiCl/20 mM Tris, pH 7.4, and dH₂O. The immunoprecipitates were then resuspended in 30 μ l of 20 mM Tris, pH 7.4, 10 mM MnCl₂ with 10 μ Ci γ -[³²P]ATP, and incubated at room temperature for 10 min, before washing in dH₂O and analyzing by one- or two-dimensional gel electrophoresis and autoradiography.

Phosphoamino acid analysis was carried out essentially as described (28). In vitro phosphorylated products identified by autoradiography were excised, the protein electroeluted in 0.05 M NH₄HCO₃, 0.1% SDS, and trypsin digested. After acid hydrolysis the sample was analyzed by two-dimensional thin layer chromatography and the location of the radioactive species was compared to the position of phosphoamino acid standards that were visualized by ninhydrin.

Two-dimensional NEPHGE-SDS Gel Electrophoresis and Immunoblotting. Two-dimensional nonequilibrium pH gel electrophoresis-SDS gel electrophoresis was carried out under standard conditions (29). Immunoprecipitates were incubated in sample buffer containing 9.5 M urea, 2% NP-40, 1.6% ampholines, pH 5–8, 0.4% ampholines, pH 3.5–10, and 5% 2-mercaptoethanol at room temperature and then electrophoresed for 6 h at 500 V in a 12.5 cm gel (4% acrylamide, 9.2 M urea, 2% NP-40, 2% ampholines, pH 3.5–10). The samples were analyzed in the second dimension by SDS-PAGE after equilibration of the first-dimension gels in SDS-sample buffer.

For immunoblotting analysis immunoprecipitates were separated by one- or two-dimensional gel electrophoresis, transferred to nitrocellulose membranes, and blocked with 3% BSA, 5% dry milk, 10 mM Tris, pH 7.9, 150 mM NaCl, and 0.05% Tween. Blots were incubated with anti-phosphotyrosine antibody followed by secondary antibody conjugated with alkaline phosphatase and then developed with bromo-chloro-indolyl phosphate and nitroblue tetrazolium.

Calcium Assay. Cells were loaded with the calcium binding fluorescent dye Indo-1 (Molecular Probes, Inc., Eugene, OR) at 1 μ M, washed extensively, and resuspended at 10^6 cells/ml. A spectrofluorimeter with a water jacketed cuvette was used to measure fluorescence levels at 37°C before and after stimulation with saturating concentrations of antibody. The TCR was stimulated with anti-Ti antibody C305 at a 1:10,000 dilution of ascites, and the CD8 ϵ chimera was stimulated with 1 μ g/ml anti-CD8 antibody OKT8 and 3 μ g/ml rabbit anti-mouse antibody. Calcium concentrations were determined using a K_d of 250 nM for calcium binding to indo-1 (30).

Results

Tyrosine Kinase Activity is Associated With the CD3 Chains. Previous work has shown that a tyrosine kinase activity is

found associated with immunoprecipitates of the ζ chain of the TCR (12, 18). We examined the possibility that the CD3 chains independently associated with a tyrosine kinase activity. The CD3 chains were immunoprecipitated from lysates of unstimulated, or anti-TCR-stimulated, Jurkat T cells prepared with 1% NP-40 detergent under conditions that dissociate the CD3 and ζ chains. To assess kinase activity the immunoprecipitates were incubated with γ -[32 P]ATP and analyzed on SDS polyacrylamide gels (Fig. 1). Strong kinase activity was observed with major products of 23–25 kD, the approximate size of the CD3 subunits, as well as a number of higher molecular mass proteins. This kinase activity is independent of the ζ subunits since ζ is not detected as a phosphorylated product, and it could not be detected in the CD3 immunoprecipitates by blotting (data not shown). Immunoprecipitates formed with normal mouse serum (lane 7), or with antibodies directed against MHC class I or CD28, had little associated kinase activity (data not shown; [18]).

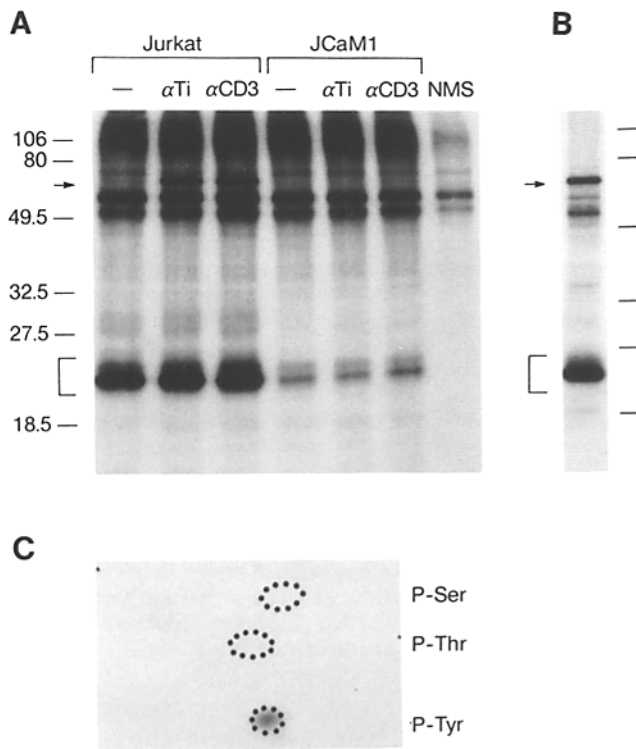


Figure 1. (A) Kinase activity associated with CD3 immunoprecipitates from Jurkat and JCaM1. The CD3 chains were immunoprecipitated from NP-40 lysates of cells stimulated for 2 min at 37°C with anti-Ti or anti-CD3 antibodies, or left unstimulated. Immunoprecipitates were incubated with γ -[32 P]ATP and analyzed by SDS-PAGE. (B) Kinase activity associated with CD3 chains immunoprecipitated from the cell surface. Jurkat cells were stimulated by coating with anti-CD3 antibody at 4°C then shifting to 37°C for 2 min. CD3 was immunoprecipitated from cell lysates using the stimulating antibody which remained bound to CD3. Immunoprecipitates were incubated with γ -[32 P]ATP and analyzed by SDS-PAGE. (C) Phosphoamino acid analysis of the in vitro phosphorylated 20–23-kD product from surface CD3 immunoprecipitates. The phosphorylated 20–23-kD product was electroeluted from SDS-PAGE and analyzed for phosphoamino acid content as described in Materials and Methods. The position of the phosphoamino acid standards is indicated by the dashed circles.

Stimulation of the TCR via the β chain or the CD3 chains slightly enhanced the associated kinase activity and resulted in the appearance of a new substrate of \sim 70 kD (lanes 2 and 3). This product is the same size as the recently identified ζ associated kinase, ZAP-70 (18). Examination of CD3 chains immunoprecipitated from the cell surface also showed associated kinase activity, and the presence of a 70-kD substrate (Fig. 1 B). Phosphoamino acid analysis of the in vitro phosphorylated 23–25-kD products from surface CD3 immunoprecipitates indicated that the associated kinase activity was specific for tyrosine residues (Fig. 1 C).

In previous work we found that the tyrosine kinase activity associated with ζ was greatly reduced in a Jurkat-derived TCR signaling mutant, JCaM1, which lacks the lck tyrosine kinase (16). Examination of the CD3-associated kinase activity in JCaM1 showed that it was also greatly reduced compared to the Jurkat line (Fig. 1 A). Stimulation of JCaM1 with anti-TCR β fails to induce signaling events and also fails to enhance CD3-associated kinase activity or association of the 70-kD protein. Although stimulation with the anti-CD3 antibody 235 can induce some weak signaling events in JCaM1, there was only a very slight enhancement of CD3-associated kinase activity and association of the 70-kD protein. These findings indicate that the CD3 subunits associate with a tyrosine kinase activity, and that TCR stimulation induces the association and/or phosphorylation of a 70-kD protein. Furthermore, the kinase activity associated with the CD3 chains appears to be in part dependent upon expression of the lck tyrosine kinase as similarly shown with the ζ chain.

To further examine the identity of the in vitro substrates of the CD3 associated kinase activity the reaction products were analyzed by two-dimensional nonequilibrium pH gel electrophoresis (NEPHGE)¹ SDS-polyacrylamide gel electrophoresis (Fig. 2 A). The migration of the \sim 23-kD phosphoproteins was consistent with the migration of the CD3 δ and ϵ subunits (25), while the more acidic \sim 25-kD products could represent CD3 chains with decreased isoelectric point and mobility due to differential glycosylation. In addition, the products appeared as twin spots possibly representing singly and doubly phosphorylated subunits. To confirm the identity of these proteins the indicated radiolabeled species (a, b, c) were excised, electroeluted, and reimmunoprecipitated with antibodies against the CD3 δ and ϵ chains (Fig. 2 B). The 25-kD spots and the more acidic 23-kD spots could be immunoprecipitated with anti- δ antibody while the more basic 23-kD spots were immunoprecipitated primarily with anti- ϵ antibody. In some gels a \sim 45-kD tyrosine phosphoprotein which migrated at the same isoelectric point as ϵ was detected and probably represents disulfide-linked ϵ dimers that were not reduced in the second dimension gel. Although the CD3 γ chain is present in the immunoprecipitates, and some phospho- γ can be detected, it appears to be a relatively poor substrate for the CD3-associated kinase (data not shown).

¹ Abbreviation used in this paper: NEPHGE, nonequilibrium pH gel electrophoresis.

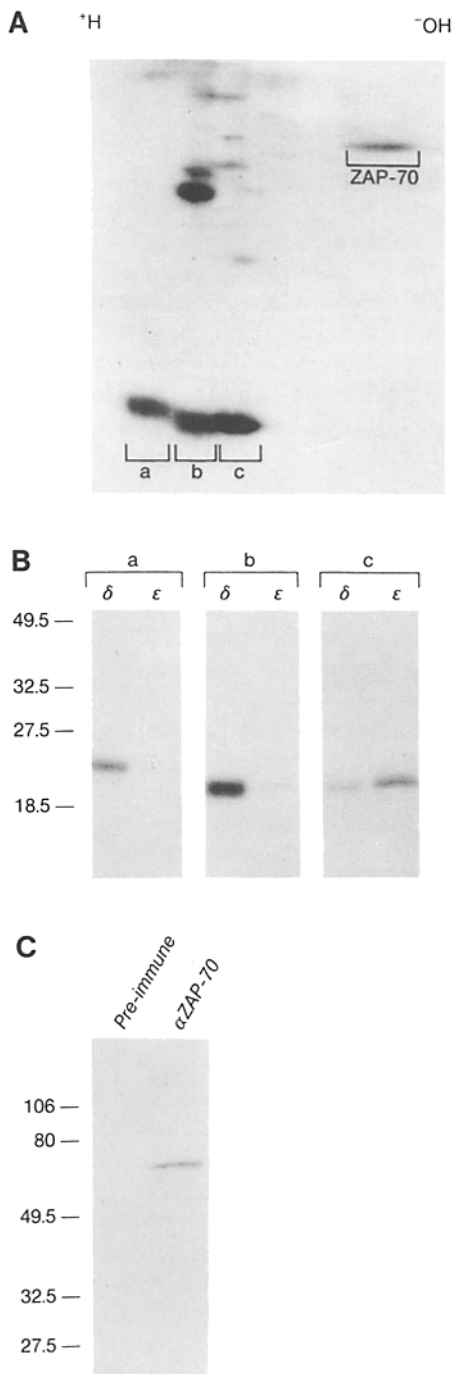


Figure 2. Analysis of the in vitro substrates of CD3-associated kinase activity by two-dimensional gels and immunoprecipitation. (A) NEPHGE-SDS gel analysis of in vitro phosphorylated CD3 immunoprecipitates. Immunoprecipitates were phosphorylated in vitro with γ - ^{32}P ATP and analyzed by on NEPHGE-SDS gels as described in Materials and Methods. (B) Immunoprecipitation of in vitro substrates (a, b, and c) with antibodies against the CD3 δ and CD3 ϵ . The indicated substrates (a, b, and c) were purified from a two-dimensional gel by electroelution and subjected to immunoprecipitation with anti-CD3 δ (SP64) and anti- ϵ (Leu4) (C). Immunoprecipitation of the 70-kD substrate from in vitro phosphorylated CD3 precipitates with anti-ZAP-70 antiserum. The 70-kD product of in vitro phosphorylated CD3 immunoprecipitates from stimulated cells was purified from one-dimensional SDS gels, or two-dimensional gels (data not shown), and immunoprecipitated with antiserum raised against a ZAP-70 peptide.

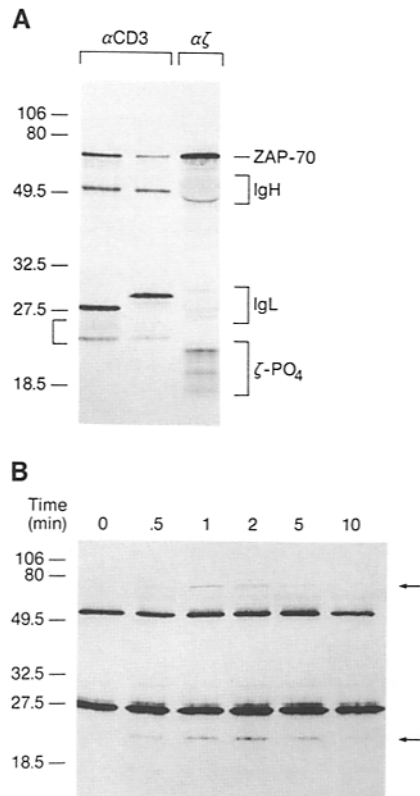


Figure 3. Association of tyrosine phosphoproteins with CD3 immunoprecipitates from stimulated cells. (A) Comparison of tyrosine phosphoproteins present in immunoprecipitates of ζ with two different CD3 immunoprecipitates. Jurkat cells were stimulated by incubating with anti-CD3 antibodies Leu4 (lane 1), or A32.1 (lane 2), or with anti-Ti antibody C305 (lane 3) at 4°C for 20 min. The cells were lysed in 1% NP-40 and surface CD3 complexes were immunoprecipitated via the stimulating antibody, or ζ was immunoprecipitated with anti-peptide antiserum. Immunoprecipitates were analyzed on 11% SDS gels and immunoblotted with anti-phosphotyrosine antibody. (B) Induction of CD3-associated tyrosine phosphoproteins following TCR stimulation. Jurkat cells were stimulated at 37°C for the indicated times with anti-Ti antibody C305. CD3 complexes were immunoprecipitated, analyzed on SDS gels, and immunoblotted with anti-phosphotyrosine antibody. The arrows indicate the 23-kD and 70-kD inducible tyrosine-phosphoproteins. The weak bands seen at \sim 18 kD and \sim 30 kD are present when the immunoprecipitating antibody alone is blotted. Reprobing the blot with anti-CD3 antibody indicated that equivalent amounts of CD3 were present in each lane (data not shown).

Reprecipitation analysis was also used to determine whether the 70-kD phosphoprotein product of the in vitro kinase reactions was the same as the recently identified ZAP-70 tyrosine kinase. Anti-ZAP-70 peptide antiserum was capable of immunoprecipitating the 70-kD phosphoprotein electroeluted from one-dimensional gels (Fig. 2 C), and two-dimensional gels (data not shown). Additionally, the apparent isoelectric point of the 70-kD protein is approximately the same as purified ZAP-70 (A. Chan, personal communication). These findings show that the CD3 δ and ϵ subunits are substrates for the CD3-associated kinase activity, and that the ZAP-70 tyrosine kinase is present in CD3 immunoprecipitates from stimulated cells.

The CD3 Chains are Tyrosine Phosphorylated After Receptor Stimulation. Although the CD3 chains can be phosphorylated

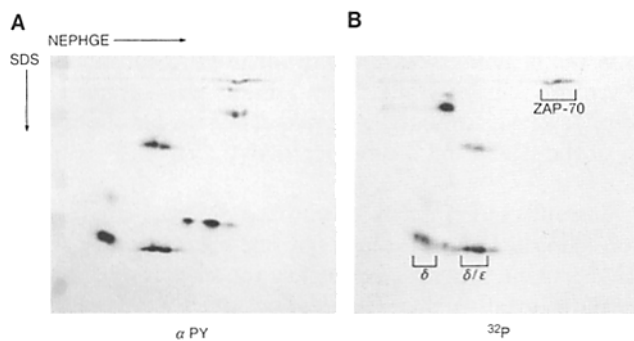


Figure 4. Comparison of CD3-associated tyrosine phosphoproteins and in vitro phosphorylated CD3 immunoprecipitates by two-dimensional gel analysis. (A) Anti-phosphotyrosine blot of CD3 immunoprecipitate resolved by two-dimensional NEPHGE-SDS gel electrophoresis. (B) Autoradiogram of the same two-dimensional gel. CD3 complexes from the cell surface were immunoprecipitated and mixed with a CD3 immunoprecipitate that had been in vitro phosphorylated with γ - ^{32}P ATP. The mixture was analyzed on a two-dimensional NEPHGE-SDS gel, transferred to nitrocellulose, blotted with anti-phosphotyrosine antibodies, and exposed to film. The location of the CD3 δ and ϵ chains, and ZAP-70, as described in Fig. 2, is indicated in the autoradiogram.

in vitro by an associated tyrosine kinase activity, it was important to determine whether this also reflected events that occurred in vivo. An anti-phosphotyrosine blot of immunoprecipitates from cells stimulated with either of two anti-CD3 antibodies revealed a number of tyrosine phosphoproteins (Fig. 3 A). The 23–25-kD phosphoproteins were in the appropriate size range to be CD3 chains, and did not comigrate with phospho- ζ when they were compared with a ζ immunoprecipitate from stimulated cells. Although phospho- ζ was not present in the CD3 immunoprecipitates, a 70 kD tyrosine phosphoprotein was detected in the anti-CD3 immunoprecipitates from stimulated cells that comigrated with ZAP-70 present in ζ immunoprecipitates. A kinetic analysis showed that both the 23 kD and the 70 kD tyrosine phosphoproteins found in the CD3 immunoprecipitates were detectable within 30 s of receptor stimulation, peaked at 1–2 min, and declined by 10 min (Fig. 3 B).

To help confirm the identity of the tyrosine phosphoproteins in the CD3 immunoprecipitates we compared them to in vitro phosphorylated CD3 chains by two-dimensional gel analysis (Fig. 4). CD3 immunoprecipitates were phosphorylated in vitro using γ - ^{32}P ATP, combined with an unlabeled CD3 immunoprecipitate, analyzed by NEPHGE-SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. Comparison of the immunoblot (Fig. 4 A) and the autoradiogram (Fig. 4 B) showed that the 23- and 25 kD tyrosine phosphoproteins comigrated with the in vitro phosphorylated CD3 δ and ϵ chains, and the 70-kD phosphoprotein comigrated with ZAP-70. As described above, apparent ϵ dimers of ~ 45 kD were detected in both the immunoblot and the autoradiogram.

Tyrosine Kinase Activity and ZAP-70 Associate With a CD8-CD3 ϵ Chimera. To determine if the isolated subunits of the CD3 chains were capable of independently associating with a tyrosine kinase activity we constructed chimeras between

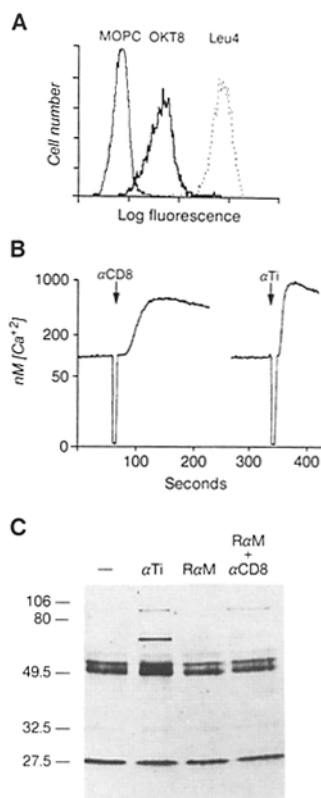


Figure 5. Expression and signaling function of the CD8- ϵ chimera. (A) Cell surface expression of CD8- ϵ and CD3 on Jurkat cells transfected with the chimera. Cells were stained with antibodies against the chimera (OKT8) or CD3 (Leu4), and FITC conjugated goat anti-mouse antibody. MOPC195 antibody (MOPC) was used as a control for nonspecific staining. Fluorescence was measured by flow cytometry. (B) Intracellular calcium levels in Jurkat transfected with CD8- ϵ after antibody stimulation of the CD8- ϵ chimera, or the TCR. The transfectant was loaded with the calcium binding dye Indo-1, equilibrated at 37°C in a spectrofluorimeter, and stimulated with anti-CD8 (OKT8) and rabbit anti-mouse antibody, or anti-TCR antibody (C305). Treatment of cells with rabbit anti-mouse antibody alone did not increase calcium levels (data not shown). (C) Induction of tyrosine phosphoproteins in the Jurkat transfectant after stimulation of CD8- ϵ or the TCR. Cells were stimulated for 2 min at 37°C with anti-TCR antibody C305, rabbit anti-mouse antibody, or rabbit anti-mouse with anti-CD8 antibody OKT8. After lysis, phosphotyrosine containing proteins were immunoprecipitated, separated by SDS-PAGE, and immunoblotted.

the cytoplasmic domains of CD3 γ , δ , and ϵ and the extracellular and transmembrane domain of CD8. We were unable to obtain stable Jurkat transfectants expressing γ or δ chimeras but we could select transfectants with a low level of CD8- ϵ expression. The difficulty in obtaining expression of these CD8-CD3 chimeras is probably due to the presence of retention and degradation signals in the cytoplasmic domains of the CD3 chains (31). Despite the low level of CD8- ϵ expression (Fig. 5 A), stimulation of the transfectants by antibody crosslinking of the chimeric molecules led to increases in intracellular calcium (Fig. 5 B) and protein tyrosine phosphorylation (Fig. 5 C). The tyrosine phosphoproteins induced after stimulation of the chimera were similar, though weaker, than those induced after TCR stimulation, except for a ~ 70 -kD protein that appeared to be relatively poorly phosphorylated. These results confirm previous work using a Tac/ ϵ chimera, which also showed that the ϵ subunit has signaling capability (10).

To examine whether CD8- ϵ was associated with a tyrosine kinase activity the chimera was immunoprecipitated from the surface of the Jurkat transfectant and assayed for the presence of kinase activity with γ - ^{32}P ATP (Fig. 6 A). We observed the phosphorylation of a 30-kD protein which is the correct size to be the CD8- ϵ chimera. The CD8 sequences present in CD8- ϵ are not responsible for this effect since im-

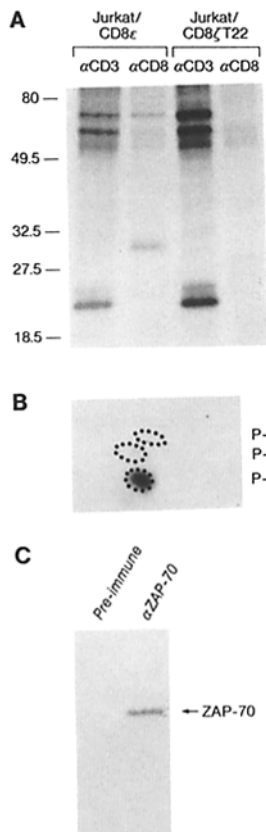


Figure 6. The ZAP-70 tyrosine kinase associates with the CD8- ϵ chimera. (A) Kinase activity associated with immunoprecipitates of the CD8- ϵ . (B) Phosphoamino acid analysis of in vitro phosphorylated CD8- ϵ . (C) Reprecipitation of in vitro phosphorylated ZAP-70 from CD8- ϵ immunoprecipitates. Jurkat cells transfected with the CD8- ϵ chimera or a truncated form of the CD8- ζ (T22) (8) were incubated with anti-CD8 or anti-CD3 antibody at 4°C and then shifted to 37°C for 2 min. After lysis the receptors expressed on the cell surface were immunoprecipitated and incubated with γ -[32 P]ATP. Products of the in vitro phosphorylation were analyzed by SDS-PAGE. Arrows indicate the position of the CD8 ϵ chimera at 30 kD and ZAP-70 at ~70 kD. Cell surface expression of the CD8- ζ T22 chimera is approximately sixfold greater than CD8- ϵ as determined by fluorescent antibody staining and flow cytometry (data not shown). Two-dimensional phosphoamino acid analysis, described in Materials and Methods, was performed on in vitro phosphorylated CD8 ϵ chimera purified by electroelution. Anti-ZAP-70 peptide antiserum, or preimmune serum, was used to reprecipitate the 70-kD substrate that had been purified by electroelution.

munoprecipitates of a truncated CD8/ ζ chimera (8) had a relatively low level of associated kinase activity which did not phosphorylate a 30-kD substrate. The failure to detect phosphorylated CD3 chains in the CD8- ϵ immunoprecipitate indicates that the presence of kinase activity is not due to association of the chimera with the TCR. Phosphoamino acid analysis showed that the kinase activity that phosphorylated the CD8- ϵ chimera was specific for tyrosine residues (Fig. 6 B). In addition to the chimera, a 70-kD protein was detected as a specific product of the immunoprecipitate kinase reaction. As observed in CD3 immunoprecipitates, the electroeluted 70-kD protein could be immunoprecipitated with anti-ZAP-70 peptide antiserum (Fig. 6 C). These findings show that the cytoplasmic domain of CD3 ϵ is sufficient for association with tyrosine kinase activity and in particular the ZAP-70 tyrosine kinase.

Discussion

To investigate the role of CD3 in signal transduction through the TCR we have focused on CD3-associated tyrosine kinase function. We, and others (32), have found that tyrosine kinase activity is associated with immunoprecipitates of the CD3 components of the TCR. Although the ζ subunit of the TCR also associates with tyrosine kinase activity, this does not explain the activity found in CD3 immunoprecipitates since ζ does not remain complexed with

CD3 under the conditions used for immunoprecipitation. The CD3 ϵ and δ subunits were the major in vitro substrates of the tyrosine kinase, while the γ subunit was only weakly phosphorylated. Similarly, we could detect the phosphorylation of the CD3 ϵ and δ subunits in vivo after TCR stimulation.

Our results indicate that at least two different kinases are involved in the CD3 associated tyrosine kinase activity. The JCaM1 mutant, which is deficient in the lck tyrosine kinase (16), shows greatly reduced levels of kinase activity associated with the CD3 chains. Similarly, kinase activity associated with the ζ chain is also greatly reduced in this mutant. These findings indicate that lck is required for association of kinase activity with the TCR. However, this does not show that lck is the kinase that is directly responsible for the associated activity. While lck has been reported to associate with the CD3 chains in the HPB.ALL cell line (33), this has been difficult to demonstrate in other cell lines, including Jurkat. Lck may instead regulate the association of other kinases with the TCR (19, 34).

In addition to lck, our results indicate that the ZAP-70 tyrosine kinase is also involved in the CD3-associated kinase activity. We were able to demonstrate that ZAP-70 is present in CD3 immunoprecipitates from stimulated cells by reprecipitation with anti-ZAP-70 antiserum (Fig. 2). ZAP-70 was initially identified through its association with the ζ chain of the TCR after receptor stimulation (18). Cotransfection studies with Cos cells have shown that the lck and fyn kinases are capable of regulating the association of ZAP-70 with the cytoplasmic domain of ζ (19). Recent work indicates that lck is responsible for the tyrosine phosphorylation of ζ , which in turn recruits ZAP-70 via phosphotyrosine-SH2 domain interactions (Iwashima, M. and A. Weiss, manuscript submitted for publication). A similar mechanism probably regulates ZAP-70 association with CD3 given the same requirement for lck function.

The significance of the association of tyrosine kinase activity with the CD3 chains was suggested by the observation that CD3 δ and ϵ were tyrosine phosphorylated following stimulation of the TCR. Tyrosine phosphorylated ZAP-70 was also observed in association with the CD3 chains after stimulation. The identity of the anti-phosphotyrosine reactive proteins in the CD3 immunoprecipitates was established by their comigration with in vitro phosphorylated CD3 chains and ZAP-70 in two-dimensional gels. The induction of CD3 tyrosine phosphorylation occurred rapidly and transiently following TCR stimulation; peaking at 1-2 min and declining by 10 min. A recent report has described the tyrosine phosphorylation of CD3 chains following antigen stimulation of a murine T cell clone (35). Similar to our findings with Jurkat, the tyrosine phosphorylation of CD3 in the murine clone was found almost entirely on the δ and ϵ subunits. This is consistent with the result that the γ subunit is also poorly phosphorylated by the CD3-associated kinase activity in vitro. The difference in phosphorylation between the δ and γ subunits is surprising given their high level of sequence identity and suggests that they may have distinct functions within the TCR.

We constructed a chimera fusing the cytoplasmic domain of CD3 ϵ to the extracellular and transmembrane domains of CD8 to determine if the cytoplasmic domain of ϵ could associate with tyrosine kinase activity independently of the other CD3 chains. Although the chimera was expressed only at low levels, it was still capable of initiating signal transduction events such as increases in intracellular calcium levels and increases in tyrosine phosphorylation of several proteins following antibody crosslinking. This result supports previous work showing that a Tac-CD3 ϵ chimera was capable of generating signals leading to IL2 production. Immunoprecipitation of the CD8/CD3 ϵ chimera revealed that it was capable of associating with tyrosine kinase activity in the absence of the endogenous CD3 chains. Furthermore, ZAP-70 was found to associate with the chimera as demonstrated by reprecipitation with anti-ZAP-70 antiserum. These findings show that the cytoplasmic domain of CD3 ϵ contains the information required for association with tyrosine kinase activity and ZAP-70. Analysis of sequences in ζ indicate that the signaling motif which is shared with other receptor proteins is sufficient for ZAP-70 association (8). Since the CD3 δ and γ chains also contain this signaling motif they may share with ϵ and ζ the ability to associate with ZAP-70. However, we could not test this due to the failure of fusions between CD8 and these CD3 chains to be expressed on the cell surface.

Both ζ and the CD3 components of the TCR become tyrosine phosphorylated following stimulation of the receptor. Surprisingly, tyrosine phosphorylation of the Tac- ϵ chimera appears to be quite weak, but substitution of the tyrosine residues with phenylalanine does eliminate signaling function (10). Tyrosine phosphorylation of the CD8- ϵ chimera has also been hard to detect, although the low level of expression of this chimera compromises interpretation of this result. While the role of tyrosine phosphorylation of the TCR is not yet clear it may serve to recruit SH2 containing proteins such as ZAP-70, and also other regulatory molecules that are then phosphorylated leading to the generation of downstream signals.

Analysis of a number of cell lines and chimeric molecules indicates that both the ζ and CD3 components of the TCR are competent to initiate signaling events leading to IL-2 production (4, 5, 9, 10). The signaling function of ζ and

CD3 ϵ have been mapped to a short stretch of amino acids which are shared with a number of signaling molecules (7, 8, 10). Furthermore the signaling events associated with ζ and CD3 are similar. Both components induce tyrosine phosphorylation of cellular proteins and activate the inositol phosphate pathway as measured by increases in intracellular calcium. In addition, both the ζ and CD3 chains are tyrosine phosphorylated following receptor stimulation, associate with the ZAP-70 tyrosine kinase, and depend upon the lck tyrosine kinase for in vitro associated kinase activity. However, differences have been observed between CD3 and ζ signaling. The pattern of tyrosine phosphoproteins induced by stimulation of a Tac- ϵ chimera appears to be distinct from the pattern seen following stimulation of a Tac- ζ chimera (10). In addition, the ability of the Thy-1 and Ly-6 accessory molecules to signal depends upon the presence of a functional ζ chain, but not CD3 (9). The similarity of the kinase activities associated with ζ and CD3 suggest that the apparent differences in tyrosine phosphoprotein induction, or accessory molecule function, are due to the recruitment of distinct substrates by the two TCR components. However, it is also possible that additional tyrosine kinases associate uniquely with either ζ or ϵ . In the case where the CD3 and ζ signaling mechanisms are redundant, the distinctions in signaling function which have been observed could be due to quantitative rather than qualitative differences. Each ζ chain contains three copies of the signaling motif while the individual CD3 chains only contain one. Multimers of the signaling motif have been shown to have greater signaling function than monomers (8). Finally, CD3 signaling may play a distinct role in early thymocyte development. Recent evidence suggests that the β chain of the TCR can be expressed on the cell surface as a homodimer in association with some of the CD3 chains prior to α chain expression (36, 37). However the ζ subunit is only weakly associated with this potential pre-T cell receptor. Signaling through the CD3 components, rather than ζ , may provide a unique and critical function in thymic development. However, more definitive studies are still required to assess whether individual activation motifs within the CD3 and ζ chains have identical or distinct functions.

We would like to thank A. Chan, S. M. Fu, C. Terhorst, R. Klausner, and L. Samelson for providing antibodies, and B. Irving for cells expressing CD8/ ζ T22.

This investigation was supported in part by a California Division-American Cancer Society Senior Fellowship, S-21-92.

Address correspondence to Dr. D. Straus, Department of Medicine, University of California-San Francisco, Box 0724, 3RD and Parnassus Aves., San Francisco, CA 94143.

Received for publication 11 June 1993.

References

1. Clevers, H., B. Alarcon, T. Willeman, and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6:629-662.
2. Weiss, A. 1988. T lymphocyte activation, in *Fundamental Im-*

- munology, 2nd ed. W.E. Paul, editor. Raven Press, New York. 359-384.
3. Sussman, J.J., J.S. Bonifacino, J. Lippincott-Schwartz, A.M. Weissman, T. Saito, R.D. Klausner, and J.D. Ashwell. 1988. Failure to synthesize the T cell CD3- ζ chain: structure and function of a partial T cell receptor complex. *Cell*. 52:85.
 4. Irving, B., and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor ζ chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*. 64:891.
 5. Romeo, C., and B. Seed. 1991. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell*. 64:1037.
 6. Reth, M. 1989. Antigen receptor tail clue. *Nature (Lond.)*. 338:383.
 7. Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor ζ chain. *Cell*. 68:889-897.
 8. Irving, B.A., A.C. Chan, and A. Weiss. 1993. Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. *J. Exp. Med.* 177:1093.
 9. Wegener, A.-M.K., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton, and B. Malissen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell*. 68:83.
 10. Letourneur, F., and R.D. Klausner. 1992. Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 ϵ . *Science (Wash. DC)*. 255:79.
 11. 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.
 12. 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.
 13. Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{fyn}. *Cell*. 70:751.
 14. 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.
 15. 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.
 16. 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.
 17. 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.
 18. 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.
 19. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell*. 71:649.
 20. Goldsmith, M.A., and A. Weiss. 1987. Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor. *Proc. Natl. Acad. Sci. USA*. 84:6879.
 21. Gold, D.P., J.M. Puck, C.L. Petty, M. Cho, J. Coligan, J.N. Woody, and C. Terhorst. 1986. Isolation of cDNA clones encoding the 20K non-glycosylated polypeptide chain of the human T-cell receptor/T3 complex. *Nature (Lond.)*. 321:431.
 22. Clevers, H.C., S. Dunlap, T.E. Wileman, and C. Terhorst. 1988. Human CD3- ϵ gene contains three minixons and is transcribed from a non-TATA promoter. *Proc. Natl. Acad. Sci. USA*. 85:8156.
 23. Weiss, A., and J.D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160:1284.
 24. Hara, T., and S.M. Fu. 1985. Human cell activation: I. Monocyte-independent activation and proliferation induced by anti-T3 monoclonal antibodies in the presence of tumor promoter 12-o-Tetradecanoyl Phorbol-13-acetate. *J. Exp. Med.* 161:641.
 25. Pessano, S., H. Oettgen, A.K. Bhan, and C. Terhorst. 1985. The T3/T cell receptor complex: antigen distinction between the two 20-kd T3 (T3- δ and T3- ϵ) subunits. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:337.
 26. Orloff, D.G., S.J. Frank, F.A. Robey, A.M. Weissman, and R.D. Klausner. 1989. Biochemical characterization of the η chain of the T-cell receptor: a unique subunit related to ζ . *J. Biol. Chem.* 264:14812.
 27. Drucker, B., T. Mamon, and T. Roberts. 1989. Oncogenes, growth factors, and signal transduction. *N. Engl. J. Med.* 321:1383.
 28. Hunter, T., and B.M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA*. 77:1311.
 29. O'Farrell, P.H. 1975. High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007.
 30. Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca⁺⁺ indicators with greatly improved fluorescent properties. *J. Biol. Chem.* 260:3440.
 31. Letourneur, F., and R.D. Klausner. 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell*. 69:1143.
 32. Wange, R.L., T.A.-N. Kong, and L. Samelson. 1992. A tyrosine-phosphorylated 70-kDa protein binds a photoaffinity analogue of ATP and associates with both the ζ chain and CD3 components of the activated T cell antigen receptor. *J. Biol. Chem.* 267:11685.
 33. Burgess, K.E., A.D. Odysseos, C. Zalvan, B.J. Drucker, P. Anderson, S.E. Schlossman, and C.E. Rudd. 1991. Biochemical identification of a direct physical interaction between the CD4:p56lck and Ti(TCR)/CD3 complexes. *Eur. J. Immunol.* 21:1663.
 34. Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell*. 73:209.
 35. Qian, D., I. Griswold-Prenner, M.R. Rosner, and F. Fitch. 1993. Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation. *J. Biol. Chem.* 268:4488.
 36. Punt, J.A., R.T. Kubo, T. Saito, T.H. Finkel, S. Kathiresan, K. Blank, and Y. Hashimoto. 1991. Surface expression of a T cell receptor β (TCR- β) chain in the absence of TCR- α , - δ , and - γ proteins. *J. Exp. Med.* 174:775.
 37. Groettrup, M., A. Baron, G. Griffiths, R. Palacios, and H. von Boehmer. 1992. T cell receptor (TCR) β chain homodimers on the surface of immature but not mature α , γ , δ chain deficient T cell lines. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2735.