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# Constitutive Tyrosine Phosphorylation of the T-Cell Receptor (TCR) ζ Subunit: Regulation of TCR-Associated Protein Tyrosine Kinase Activity by TCR ζ

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**The T-cell receptor (TCR) ζ subunit is an important component of the TCR complex, involved in signal transduction events following TCR engagement. In this study, we showed that the TCR ζ chain is constitutively tyrosine phosphorylated to similar extents in thymocytes and lymph node T cells. Approximately 35% of the tyrosine-phosphorylated TCR ζ (phospho ζ) precipitated from total cell lysates appeared to be surface associated. Furthermore, constitutive phosphorylation of TCR ζ in T cells occurred independently of antigen stimulation and did not require CD4 or CD8 coreceptor expression. In lymph node T cells that constitutively express tyrosine-phosphorylated TCR ζ, there was a direct correlation between surface TCR-associated protein tyrosine kinase (PTK) activity and expression of phospho ζ. TCR stimulation of these cells resulted in an increase in PTK activity that coprecipitated with the surface TCR complex and a corresponding increase in the levels of phospho ζ. TCR ligations also contributed to the detection of several additional phosphoproteins that coprecipitated with surface TCR complexes, including a 72-kDa tyrosine-phosphorylated protein. The presence of TCR-associated PTK activity also correlated with the binding of a 72-kDa protein, which became tyrosine phosphorylated in vitro kinase assays, to tyrosine phosphorylated TCR ζ. The cytoplasmic region of the TCR ζ chain was synthesized, tyrosine phosphorylated, and conjugated to Sepharose beads. Only tyrosine-phosphorylated, not nonphosphorylated, TCR ζ beads were capable of immunoprecipitating the 72-kDa protein from total cell lysates. This 72-kDa protein is likely the murine equivalent of human PTK ZAP-70, which has been shown to associate specifically with phospho ζ. These results suggest that TCR-associated PTK activity is regulated, at least in part, by the tyrosine phosphorylation status of TCR ζ.**

The αβ T-cell receptor (TCR) is composed of the antigen-binding clonotypic αβ TCR heterodimer associated with the CD3 γ, CD3 δ, and CD3 ε subunits (10, 24) and the alternatively spliced TCR ζ and η gene products (2, 9, 34, 62). The five TCR-CD3 invariant chains (γ, δ, ε, ζ, and η) possess intracytoplasmic domains that are proposed to make up the signalling apparatus of the TCR (10, 25). Stimulation of the TCR by an antigen or by monoclonal antibodies results in rapid activation of two closely linked signal transduction pathways, the protein tyrosine kinase (PTK) pathway and the phosphatidyl inositide pathway (reviewed in reference 25). The TCR-mediated activation of PTKs results in the tyrosine phosphorylation of several proteins, including the TCR ζ chain (15, 42) and other CD3 components (36), the protooncogene *vav* (29), PTK ZAP-70 (7), and phospholipase Cγ1 (35, 44, 61). The tyrosine phosphorylation of phospholipase Cγ1 augments phospholipase C catalytic activity, leading to the generation of inositol lipid-derived second messengers (4, 37).

Several experimental systems have been developed to fully define the roles of individual TCR-CD3 components in T-cell activation events. For example, chimeric proteins made by linking the extracellular domains of either CD8, CD4, or the Tac antigen (CD25) with the cytoplasmic domain of TCR ζ and/or CD3 ε have been expressed in cell lines independently of the other TCR-CD3 subunits (18, 19, 27, 28, 39). Antibody stimulation of these chimeric constructs results in a cascade of intracellular signals normally transduced by the intact TCR-CD3 complex. Such studies have demonstrated that both TCR ζ and CD3 ε can function as autonomous signal transduction modules (28, 60). The signalling functions of the CD3 ε and TCR ζ components appear to reside within a common sequence motif comprising two critically spaced tyrosine residues (18, 28, 39). Since the TCR-CD3 subunits possess no identifiable kinase activity, the TCR-CD3 complex is proposed to interact with one or more cytoplasmic PTKs. Two members of the *src* family of PTKs, $p59^{fyn}$ and $p56^{lck}$, have been implicated in TCR signalling events. Under low-stringency conditions, $p59^{fyn}$ has been shown to coprecipitate with the TCR-CD3 complex (43). Overexpression of $p59^{fyn}$ in transgenic mice (11) and expression of a constitutively active form of $p59^{fyn}$ in T-cell hybridomas significantly enhances TCR signal transduction (12). PTK $p56^{lck}$, which associates with the CD4 and CD8 coreceptor molecules, is also involved in TCR signalling (40, 52–54). Two mutant T-cell lines which lack $p56^{lck}$ are defective in TCR-mediated signalling (20, 46). Reconstitution of these lines with $p56^{lck}$ restores the capacity of the cells to transduce TCR-mediated signals, independently of coreceptor expression. Recent experiments with a human T-cell line have identified a novel PTK, termed ZAP-70, which specifically associates with the tyrosine-phosphorylated form of the TCR ζ chain following TCR stimulation (7, 8). A 70-kDa PTK has also been demonstrated to associate with the CD3 ε subunits of the activated TCR complex (58). Following TCR engagement, up to six potential tyrosine residues in

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murine TCR ζ may become tyrosine phosphorylated by unidentified PTKs (2, 26, 34, 41). Given the finding that phosphorylation of the TCR ζ chain is correlated with T-cell activation and is required for the association of ZAP-70, regulation of TCR ζ phosphorylation is likely to play an essential role in TCR signal transduction.

Interestingly, the TCR ζ chain expressed in immature thymocytes appears to be constitutively tyrosine phosphorylated (21, 30–32, 55). This constitutive phosphorylation is proposed to reflect PTK activation resulting from in vivo receptor engagement, in mechanisms which may involve CD4-ligand interactions (5, 30–32). Such a model is consistent with the finding that cross-linking of surface CD4 in T cells results in TCR ζ phosphorylation (53). However, it has not been established how the tyrosine-phosphorylated TCR ζ subunit influences TCR signal transduction events. Furthermore, it remains to be established whether CD4 interactions in the thymus are the only means by which constitutive phosphorylation of TCR ζ is maintained.

To examine the consequences of constitutive tyrosine phosphorylation of the TCR ζ chain on TCR signal transduction further, we compared the status of TCR ζ phosphorylation in thymocytes and mature T cells from C57BL/6J mice and H-Y TCR transgenic mice (23, 49, 56) and determined how TCR-associated PTK activity may be regulated by the tyrosine phosphorylation status of TCR ζ.

## MATERIALS AND METHODS

**Mice.** C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and bred in the animal facility in the Department of Microbiology and Immunology at the University of British Columbia. *H-2*$^b$ H-Y TCR transgenic mice were produced as previously described (22, 23, 49) and bred in our own facility.

**Antibodies.** Antibodies and their specificities were as follows: F23.1, transgenic TCR β chain (3, 45); T3.70, transgenic TCR α chain (47); 145-2C11, CD3 ε (American Type Culture Collection, Rockville, Md.); 53.67, CD8α (American Type Culture Collection); GK1.5, CD4 (American Type Culture Collection); 4G10, phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, N.Y.); I3/2, CD45 (50); 141-31, H-2D$^b$ (Bioproducts for Science Inc., Indianapolis, Ind.). G3 is a monoclonal antibody (MAb) generated against the cytoplasmic domain of TCR ζ prepared by standard hybridoma technology (50a). G3 is capable of immunoprecipitating both nonphosphorylated TCR ζ and tyrosine-phosphorylated TCR ζ. Anti-p56$^{lck}$ was prepared by immunizing rabbits with a TrpE-*lck* fusion protein comprising N-terminal residues 3 to 147. Anti-p59$^{fyn}$ was purchased from Upstate Biotechnology Inc., while fluorescein-conjugated anti-CD8α (53.67) and phycoerythrin-conjugated anti-CD4 (GK1.5) antibodies were purchased from Becton-Dickinson (Mississauga, Ontario, Canada) and fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin (SaMIg-FITC) was obtained from Silenus (Hawthorne, Australia).

**Cell purification and flow cytometry.** Thymocytes and/or lymph node T cells were collected from mice at 6 to 12 weeks of age, and single-cell suspensions were prepared in RPMI 1640 medium (GIBCO, Burlington, Ontario, Canada) supplemented with 2% fetal bovine serum (lot no. 43N3092; GIBCO). B cells were depleted by addition of sheep anti-mouse immunoglobulin-coated magnetic beads (Dynabeads, no. 11006; Dynal Inc., Great Neck, N.Y.) as previously described (6). In certain experiments, thymocytes and/or lymph node T cells were depleted of CD4$^+$ cells and/or CD8$^+$ cells by incubation with anti-CD4 or anti-CD8 culture supernatants followed by depletion with Dynabeads. In typical experiments, the purified populations of thymocytes and/or lymph node T cells contained less than 1% CD4$^+$ and/or CD8$^+$ T cells and less than 0.5% B cells, respectively. Fluorescence-activated cell sorter analysis was performed as previously described (6).

**Cell stimulations, immunoprecipitations, immunoblotting, in vitro kinase assays, and phosphoamino acid analysis.** Single-cell suspensions were prepared in Iscove's modified Dulbecco's medium (GIBCO) at a concentration of $10^8$ cells per ml. Cells were stimulated for the times indicated in the figure legends by addition of anti-CD3 ε (50 μg/ml). Following the stimulation period, the cells were rapidly washed once in cold phosphate-buffered saline and the pellet was suspended in 0.5% Triton X-100 lysis buffer (20 mM Tris-Cl [pH 7.6], 150 mM NaCl, 2.0 mM EDTA, protease and phosphatase inhibitors) at a concentration of $2 \times 10^7$/ml. The cell lysates were gently mixed and placed on ice for 10 min. Total cell lysates were cleared by centrifugation at 12,000 × *g* for 10 min, and the supernatants were immunoprecipitated for CD3 ε-associated proteins as previously described (32). Surface TCR-CD3 complexes were immunoprecipitated through the stimulatory antibodies by addition of protein A-Sepharose beads (Pharmacia Fine Chemicals, Baie D'Urfé, Quebec, Canada). For detection of surface TCR-CD3 complexes in unstimulated cells, the cells were incubated with anti-CD3 ε antibodies at 4°C in the presence of 0.05% sodium azide for 15 min. Excess antibody was washed off prior to lysis in 0.5% Triton X-100 lysis buffer and precipitation with protein A beads. For immunoprecipitation of p56$^{lck}$ and p59$^{fyn}$, the polyclonal antisera were preincubated with protein A beads prior to incubation with cell lysates.

Immunoprecipitates were electrophoresed on sodium dodecyl sulfate (SDS)–13.5 or 8% polyacrylamide gel electrophoresis (PAGE) gels. The gels were then equilibrated in transfer buffer (20 mM Tris-HCl, 150 mM glycine, 20% methanol) and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore Canada Ltd., Mississauga, Ontario, Canada) at 200 mA for 1.5 h in a Transblot apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The polyvinylidene difluoride membranes were blocked in 4% bovine serum albumin in TBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) for 2 h at room temperature and then washed three times in TBS containing 0.05% Tween 20 (TTBS). The blots were washed twice in TTBS, for 10 min each time, and then incubated for 2 h with the anti-phosphotyrosine MAb (1 μg/ml) or an anti-TCR ζ MAb (2.5 μg/ml) in 1% bovine serum albumin–TTBS. The blots were washed and incubated for 45 min with goat-anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase (1/10,000 dilution in 1% bovine serum albumin–TTBS) (Southern Biotechnology Associates Inc., Birmingham, Ala.). The blots were washed three times in TTBS, for 30 min each time, and then twice in TBS, for 5 min each time. The blots were subsequently developed with the enhanced chemiluminesence assay as described in the manufacturer's instructions (ECL kit; Amersham Canada Ltd., Oakville, Ontario, Canada). In vitro kinase assays of immunoprecipitates were done essentially as previously described (7). Phosphoamino acid analysis was also done as previously described (59).

**Tyrosine-phosphorylated TCR ζ coupling to Sepharose beads.** The cytoplasmic domain of the TCR ζ chain (residues

52 to 164) was synthesized in its entirety and phosphorylated on tyrosyl residues by baculovirus-produced $p56^{lck}$ PTK as previously described (59). One hundred micrograms of nonphosphorylated or tyrosine-phosphorylated TCR ζ was coupled to activated CH-Sepharose 4B in accordance with the manufacturer's (Pharmacia LKB Biotechnology) instructions. Approximately 100 μg of protein was coupled to 100 μl of swollen beads, with an efficiency of coupling of greater than 80% under these conditions. The TCR ζ- and phospho ζ-coupled beads were used as affinity ligands to precipitate proteins from total T-cell lysates at a ratio of $10^7$ cells per 10 μl of packed beads. The lysates were incubated with the beads for 2 h, washed four times in 0.5% Triton X-100 lysis buffer, and subjected to in vitro kinase assays as previously described (7).

## RESULTS

**The TCR ζ chain is constitutively tyrosine phosphorylated in both thymocytes and peripheral lymph node T cells.** The tyrosine phosphorylation status of the TCR ζ chain isolated from normal C57BL/6J thymocytes and lymph node T cells was assessed by immunoprecipitating TCR-CD3 complexes from total cell lysates and immunoblotting them with anti-phosphotyrosine MAbs (32). As in the experiments of Nakayama et al. (30–32), we found that the TCR ζ chain is constitutively tyrosine phosphorylated in thymocytes isolated from C57BL/6J mice, migrating as a 21-kDa phosphoprotein (phospho ζ) (Fig. 1A, lanes 1 and 2) (30–32). However, in contrast to those reports, we also detected phospho ζ in TCR-CD3 immunoprecipitates from lymph node T cells (Fig. 1A, lane 4). Densitometric scans indicated that the levels of phospho ζ were approximately threefold higher in C57BL/6J lymph node T cells than in equivalent numbers of thymocytes (lane 3 versus lane 1). In addition to phospho ζ, a second, unidentified 30-kDa phosphoprotein also coprecipitated with the TCR-CD3 complex isolated from lymph node T cells (Fig. 1A, lanes 3 and 4). Given the knowledge that immature thymocytes express much less TCR than do mature T cells, the greater levels of phospho ζ in lymph node T cells may reflect the increased number of intact TCR-CD3 complexes precipitated from these cells. To examine this possibility, the blots in Fig. 1A were stripped and reprobed with an anti-TCR ζ MAb. As shown in Fig. 1B, the nonphosphorylated TCR ζ chain migrated with an apparent molecular mass of 16 to 18 kDa. It is also evident from Fig. 1B that the amount of TCR ζ immunoprecipitated from lymph node T cells was roughly threefold greater than that immunoprecipitated from equivalent numbers of thymocytes (Fig. 1B, lanes 3 versus 1 and 4 versus 2). These data suggest that in thymocytes and lymph node T cells, TCR-associated ζ chains are tyrosine phosphorylated to similar extents. The faint 21-kDa band detected in Fig. 1B, lane 4, could be due to either a residual anti-phosphotyrosine antibody or detection of phospho ζ by the anti-ζ MAb. Although the anti-ζ MAb can detect both nonphosphorylated ζ and phospho ζ, the former possibility is more likely since our preliminary studies suggested that, at most, 1% of the total TCR ζ is tyrosinse phosphorylated in ex vivo lymph node T cells (46a); the amount of phospho ζ in Fig. 1B is likely too low for detection by the anti-ζ MAb.

**In vivo tyrosine phosphorylation of the TCR ζ chain can occur in the absence of the nominal antigen.** Since it has been reported that the TCR ζ subunit becomes tyrosine phosphorylated in mature T cells following TCR engagement (15, 42), it was conceivable that the lymph node T cells analyzed in our study were antigenically stimulated in vivo. Alternatively, other regulatory events may contribute to the formation of phospho ζ. We addressed these possibilities by comparing the tyrosine phosphorylation levels of the TCR ζ chain in lymph node T cells and thymocytes isolated from H-Y TCR transgenic mice. On most of their thymocytes, these mice express an αβ TCR specific for the male H-Y peptide presented by H-$2D^b$ major histocompatibility complex class I molecules (22, 23). Mature $CD4^-$ $CD8^+$ T cells from *H-2*$^b$ female TCR transgenic mice that express the transgenic TCR comprise a population of virgin T cells, with the transgenic α chain confering reactivity to the male antigen (16, 22, 38, 48, 57). Therefore, if antigenic stimulation was required for attainment of the constitutive TCR ζ phosphorylation that we detected in C57BL/6J lymph node T

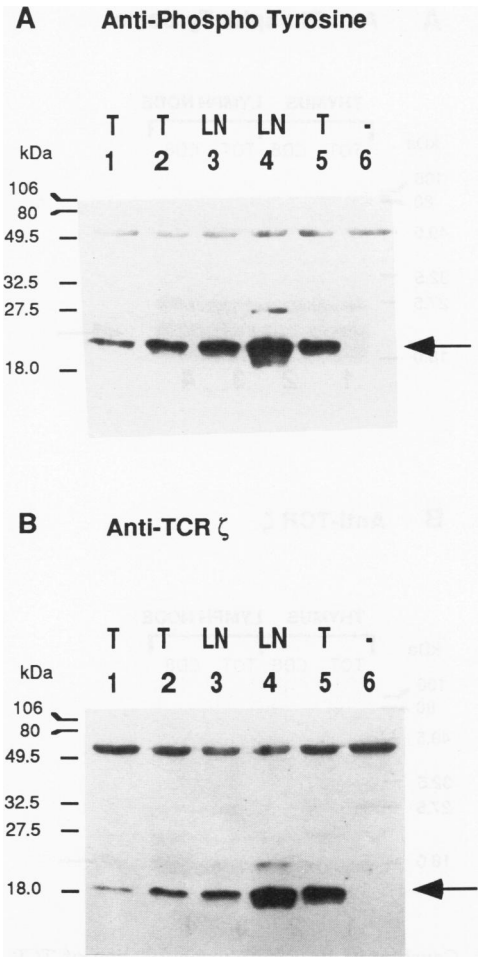


FIG. 1. Constitutive expression of tyrosine-phosphorylated TCR ζ in thymocytes (T) and lymph node T cells (LN). (A) C57BL/6J thymocytes (lanes 1, 2, and 5) and lymph node T cells (lanes 3, 4) were lysed and immunoprecipitated with an anti-CD3 ε MAb under conditions which maintain TCR ζ phosphorylation. Immune complexes were resolved on SDS–13.5% PAGE followed by immunoblotting with MAb 4G10. (B) The blots in panel A were stripped and reprobed with an anti-TCR ζ MAb. Lanes 1 and 3 contained immunoprecipitates from $10^7$ cells, lanes 2 and 4 contained immunoprecipitates from $3 \times 10^7$ cells, and lane 5 contained immunoprecipitates from $4.5 \times 10^7$ thymocytes. Lane 6 contained an antibody control. The arrows indicate the positions of tyrosine-phosphorylated (A) and nonphosphorylated (B) TCR ζ.

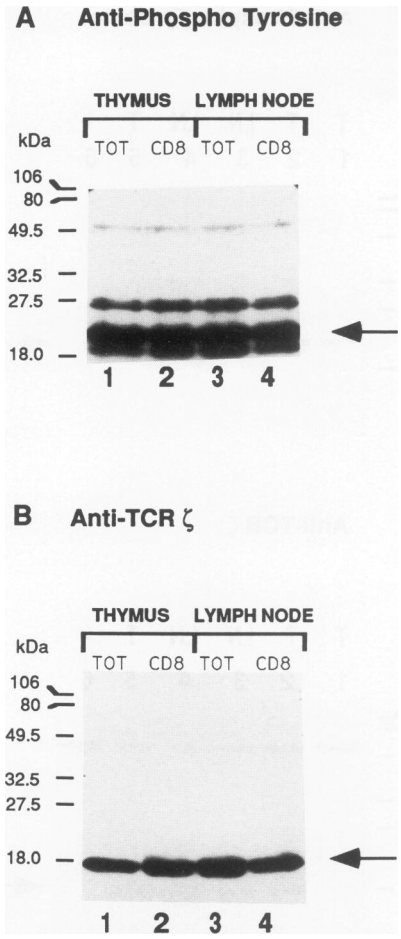


FIG. 2. Constitutive tyrosine phosphorylation of TCR ζ in the absence of male antigen and/or coreceptor expression. (A) $CD4^-$ $CD8^+$ thymocytes and lymph node T cells were isolated from female *H-2*$^b$ TCR transgenic mice, lysed, immunoprecipitated with an anti-CD3 MAb, and immunoblotted with anti-phosphotyrosine antibodies. We compared $3 \times 10^7$ unseparated thymocytes (lane 1), lymph node T cells (lane 3), $CD4^-$ $CD8^+$ thymocytes (lane 2), and lymph node T cells (lane 4) from *H-2*$^b$ female TCR transgenic for constitutive phosphorylation of the TCR ζ chain. (B) We also immunoblotted $5 \times 10^6$ cell equivalents of the samples in panel A with an anti-TCR ζ MAb. The arrows indicate the positions of tyrosine-phosphorylated (A) and nonphosphorylated (B) TCR ζ.

cells or thymocytes, little or no phospho ζ would be expected in either $CD4^-$ $CD8^+$ αβ $TCR^+$ lymph node T cells or thymocytes isolated from αβ TCR transgenic female mice. To examine this possibility, we isolated $CD4^-$ $CD8^+$ thymocytes and peripheral T cells from H-Y TCR transgenic mice. All of the $CD4^-$ $CD8^+$ cells isolated expressed the TCR β transgene product (F23.1), while 95 and 67% of the enriched $CD4^-$ $CD8^+$ populations of thymocytes and lymph node cells, respectively, expressed the α transgene product (T3.70) (22, 48, 49).

The unseparated and $CD4^-$ $CD8^+$ thymocytes and lymph node T cells from the TCR transgenic female mice were lysed, and the TCR-CD3 complexes were precipitated with an anti-CD3 ε MAb and analyzed by immunoblotting. The levels of phospho ζ expressed in these different cell populations are shown in Fig. 2A. It is evident from the results that unseparated thymocytes (lane 1) and lymph node T cells (lane 3) express a constitutively tyrosine-phosphorylated TCR ζ chain. In three independent experiments, the levels of phospho ζ ranged from roughly equivalent to twofold higher in lymph node T cells (lane 3 versus lane 1). Moreover, $CD4^-$ $CD8^+$ enriched cell populations from TCR transgenic female mice maintained levels of phospho ζ which were roughly equivalent to those of unseparated thymocytes (lane 2 versus lane 1) and lymph node T cells (lane 4 versus lane 3). The levels of total TCR ζ precipitated from these cell populations was subsequently determined by immunoblotting with an anti-TCR ζ MAb. The amount of TCR ζ precipitated from both the unseparated and $CD4^-$ $CD8^+$ lymph node T-cell populations was also roughly equivalent to that obtained from thymocyte populations (Fig. 2B, lanes 3 and 4 versus 1 and 2). It is important to note that thymocytes from TCR transgenic mice express high levels of surface TCR as a consequence of the TCR transgene, at levels approaching that detected in mature T cells (22, 23). These results demonstrate that virgin $CD4^-$ $CD8^+$ thymocytes and mature, unstimulated $CD4^-$ $CD8^+$ peripheral T cells maintain expression of phospho ζ at levels comparable to those in normal mice. Moreover, the amount of constitutively tyrosine-phosphorylated TCR ζ correlates with the levels of total precipitable TCR ζ. In $CD4^+$ $CD8^+$ thymocytes, CD4-mediated intracellular signals are reported to induce tyrosine phosphorylation of TCR ζ and degradation of newly synthesized TCR complexes within the endoplasmic reticulum (5, 32). We found that $CD4^-$ $CD8^-$ thymocytes from H-Y TCR transgenic male and female mice also expressed phospho ζ at levels equivalent to those of unseparated thymocyte populations from the same mice (data not shown). Therefore, absence of CD4 and CD8 failed to influence the overall levels of phospho ζ. These results are consistent with the interpretation that the constitutive tyrosine phosphorylation of TCR ζ may occur through regulatory mechanisms which can function independently of coreceptor expression and exposure to the male antigen.

**Tyrosine phosphorylation of multiple surface TCR-associated proteins requires TCR stimulation.** It has recently been demonstrated that in human T cells a 70-kDa PTK (ZAP-70) specifically associates with the tyrosine-phosphorylated TCR ζ chain (7, 8). Given our finding that lymph node T cells express a constitutively tyrosine-phosphorylated TCR ζ, it was of interest to determine whether any phosphoproteins such as ZAP-70 are complexed with phospho ζ in an activation-independent manner. To perform these experiments, we initially examined whether any phospho ζ was associated with the surface TCR complex. Surface TCR was precipitated from unstimulated C57BL/6J lymph node T cells and compared with the amounts of phospho ζ-TCR complexes precipitated from total cell lysates with an anti-CD3 ε MAb. As illustrated in Fig. 3A, approximately 35% of the total precipitable tyrosine-phosphorylated TCR ζ was associated with the surface TCR complex (lane 1 versus lane 5). In four independent experiments, we noted that these levels varied from as low as 25% to as high as 75%.

To study whether TCR engagement altered the levels of phospho ζ, surface TCR complexes were isolated following T-cell activation. Cell lysates were prepared from stimulated cells and incubated with protein A-coupled beads to precipitate the stimulating antibody. TCR engagement of lymph node T cells for up to 30 min resulted in a two- to threefold increase in the detection of surface TCR-associated phospho ζ (Fig. 3A, lanes 2 to 4 versus lane 1). To examine the presence of additional surface TCR-associated phosphoproteins more carefully, the immunoblots in Fig. 3A were

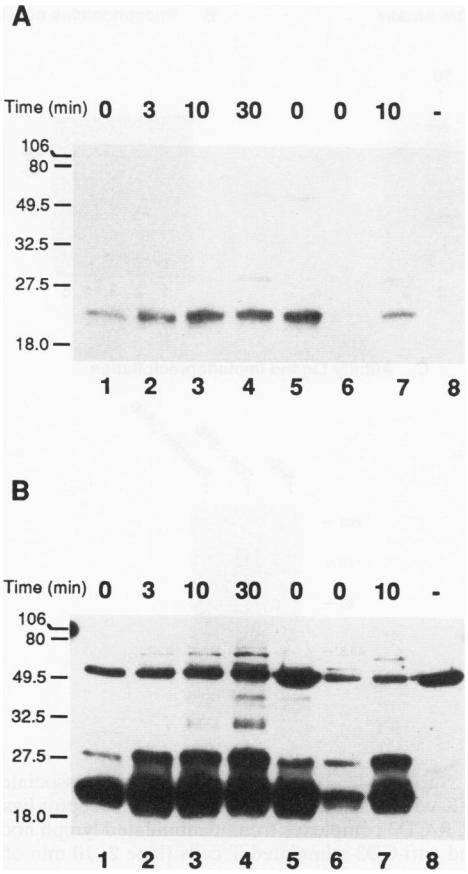


FIG. 3. The association of multiple phosphoproteins with a constitutively tyrosine-phosphorylated TCR ζ chain requires TCR stimulation. (A) Peripheral lymph node T cells were untreated (lane 1) or stimulated with an anti-CD3 MAb for 3 (lane 2), 10 (lane 3), or 30 (lane 4) min. Lymph node T cells were also cultured for 4 h at 37°C (lane 6) and subsequently stimulated with an anti-CD3 ε MAb for 10 min (lane 7). The surface-associated TCR immunoprecipitates from unstimulated and stimulated samples were analyzed by Western blotting (immunoblotting) with an anti-phosphotyrosine MAb. In lane 5, total cell lysates were immunoprecipitated and assessed for tyrosine-phosphorylated protein associations. Lanes 1 to 7 contained immunoprecipitates from $3 \times 10^7$ C57BL/6J lymph node T cells, while lane 8 contained only the antibody used for the precipitations. (B) Prolonged exposure of the immunoblots shown in panel A revealed the presence of phosphoproteins of 30, 34, 45, and 70 kDa. The numbers on the left are molecular sizes in kilodaltons.

exposed for prolonged periods. In this manner, a tyrosine-phosphorylated 30-kDa protein was found to coprecipitate with the surface TCR complex in the absence of TCR stimulation. This 30-kDa phosphoprotein appears to be similar to the 30-kDa phosphoprotein identified in Fig. 1A. In this experiment, a 72-kDa phosphoprotein was undetectable in unstimulated lymph node T cells (Fig. 3B, lane 1) although it could be detected as a very faint band in other experiments. TCR engagement increased the amounts of tyrosine-phosphorylated p30 and p72 detected approximately four- to fivefold (Fig. 3B, lanes 1 to 4). TCR stimulation also contributed to the detection of tyrosine-phosphorylated proteins with molecular masses of 45 and 34 kDa (Fig. 3B, lanes 2 to 4 versus lane 1). Many of these tyrosine-phosphorylated proteins were also detected in TCR-CD3 precipitates from total cell lysates of unstimulated cells (Fig. 3B, lane 5). Under the stimulation conditions described, the amounts of nonphosphorylated TCR ζ precipitated from the surface TCR complex were roughly equivalent before and after TCR stimulation (data not shown).

To examine how the tyrosine-phosphorylated TCR ζ chain influenced the association of coprecipitating phosphoproteins, C57BL/6J lymph node T cells were cultured in vitro for 4 h at 37°C. This led to a four- to sixfold reduction in tyrosine-phosphorylated surface-associated TCR ζ (Fig. 3B, lane 6). Subsequent TCR engagement of the cultured cells elicited tyrosine phosphorylation of 72- and 30-kDa proteins and the 21-kDa protein which is likely TCR ζ (Fig. 3B, lane 7).

**A tyrosine kinase associated with surface TCR-phospho ζ complexes is activated following TCR engagement.** Given the large number of phosphoproteins which associated with the surface TCR complex, we next examined whether surface TCR-phospho ζ complexes are associated with any PTKs. Intact surface TCR-CD3 complexes were precipitated and analyzed in kinase assays. Following the in vitro kinase assay, several phosphorylated proteins with molecular masses of 110, 72, 55, 30, and 21 kDa were detected in TCR precipitates from unstimulated lymph node T cells (Fig. 4A and 5A, lanes 1). These phosphorylated proteins are analogous to those detected in kinase assays of TCR-CD3 precipitates of total lymph node T-cell lysates (lane 3). Importantly, a marked increase in kinase activity was observed in surface TCR-CD3 precipitates following TCR engagement (Fig. 4A and 5A, lanes 2). Immunoprecipitates with anti-$D^b$ (141-30) and anti-CD45 (I 3/2) antibodies and normal rabbit serum demonstrated no significant phosphorylation of the TCR-associated phosphoproteins (Fig. 4A, lanes 7 to 9).

In both kinase assays and immunoblot analyses, we identified a 72-kDa protein which coprecipitated with the TCR complex. To examine further the possibility that surface TCR-phospho ζ complexes in lymph node T cells associate with a PTK which may be the murine equivalent of ZAP-70, lymph node T cells were cultured for several hours to dephosphorylate TCR ζ, as shown in Fig. 3A, lane 6. Upon TCR ζ dephosphorylation, we detected only minimal kinase activity in surface TCR complexes or in TCR immunoprecipitates of total cell lysates (Fig. 4A, lanes 4 and 6, respectively). Importantly, subsequent engagement of the TCR led to a dramatic increase in kinase activity, and this was correlated with TCR ζ rephosphorylation and phosphorylation of the 72-kDa protein (Fig. 4A, lane 5). It is apparent from the results in Fig. 4A and 5A that there was some variability in the amount of kinase activity that coprecipitated with surface TCR-phospho ζ in unstimulated cells (lane 1). This may reflect the variability noted in the amount of surface TCR-associated phospho ζ present in lymph node T cells, which can range from 25 to 75% of total phospho ζ. Again, TCR stimulation increased the tyrosine phosphorylation of surface-associated TCR ζ (Fig. 4B, lanes 1 to 3), while the amounts of surface-precipitable nonphosphorylated TCR ζ were fairly equivalent in unstimulated and stimulated cells (Fig. 4C, lanes 1 and 2). Five of the phosphorylated proteins indicated in Fig. 5A were examined for phosphoamino acid content. As shown Fig. 5B, all five bands were phosphorylated on tyrosine residues. The presence of phosphoserine and phosphothreonine in band 3 may be due to association of nonspecific kinase with the immunoglobulin heavy chain (data not shown).

As an additional means of determining whether the surface TCR-phospho ζ-associated PTK is the murine equivalent of ZAP-70, the cytoplasmic domain of TCR ζ was tyrosine

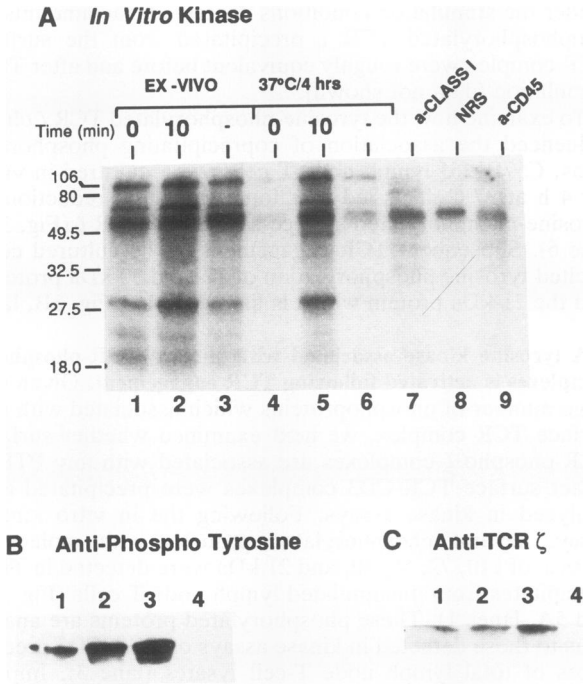


FIG. 4. Surface TCR-CD3 complexes associate with a kinase which is activated following TCR stimulation. (A) Surface TCR-CD3 complexes from $10^7$ unstimulated (lane 1) or TCR-stimulated (lane 2; 10 min of stimulation) C57BL/6J lymph node T cells and TCR-CD3 complexes precipitated from total T-cell lysates (lane 3) were subjected to in vitro kinase assays, SDS-PAGE, and autoradiography. Lymph node T cells were also cultured for 4 h at 37°C (lane 4) and subsequently stimulated with an anti-CD3 ε MAb for 10 min (lane 5) and then subjected to surface TCR-CD3 precipitation and kinase assays. TCR-CD3 immunoprecipitates and kinase assays from total cell lysates of T cells cultured for 4 h were in lane 6. Control immunoprecipitates with an anti-major histocompatibility complex class I MAb (lane 7), normal rabbit serum (NRS; lane 8), and an anti-CD45 MAb (lane 9) are shown. The numbers on the left are molecular sizes in kilodaltons. (B) Samples identical to those shown in lanes 1 to 3 of panel A were also examined by anti-phosphotyrosine immunoblot analysis subsequent to TCR-CD3 precipitations. (C) The blots in panel B were reprobed with an anti-TCR ζ MAb.

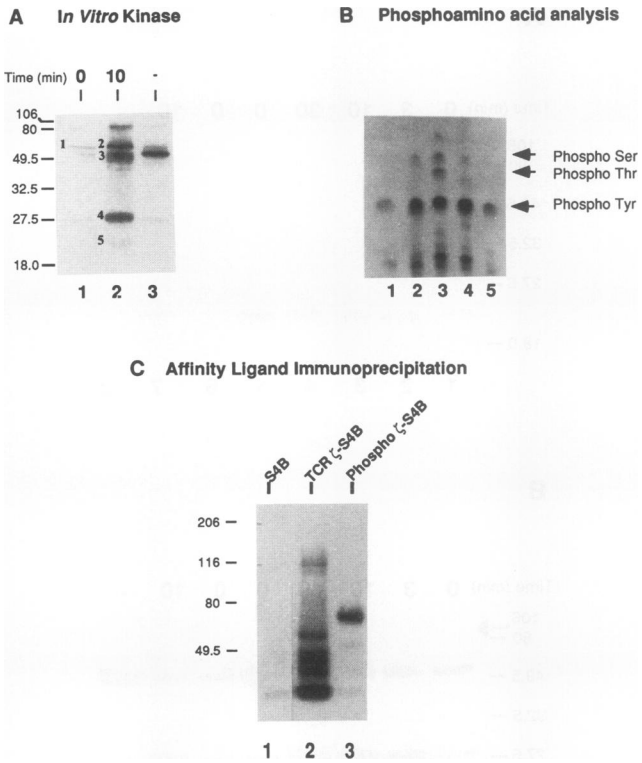


FIG. 5. Surface TCR-phospho ζ complexes associate with a 72-kDa PTK which is activated following TCR receptor ligation. (A) Surface TCR-CD3 complexes from unstimulated lymph node T cells (lane 1) and anti-CD3-stimulated T cells (lane 2; 10 min of stimulation) were precipitated and subjected to in vitro kinase assays, SDS-PAGE, and autoradiography. Lane 3 represents the TCR-CD3 complexes precipitated from total cell lysates. (B) The phosphoproteins labelled 1 to 5 in panel A were excised from the polyvinylidene difluoride membrane and subjected to phosphoamino acid analysis. (C) The cytoplasmic domain of TCR ζ was tyrosine phosphorylated in vitro and coupled to Sepharose beads. Unconjugated beads (lane 1), ζ-coupled beads (lane 2), and phospho ζ-coupled Sepharose beads (lane 3) were used to precipitate proteins from total T-cell lysates which were characterized by an in vitro kinase assay followed by SDS-PAGE and autoradiography. The numbers beside the panels are molecular sizes in kilodaltons.

phosphorylated and coupled to Sepharose beads. As shown in Fig. 5C, only tyrosine-phosphorylated (lane 3), not nonphosphorylated (lane 2), TCR ζ beads were capable of precipitating a 72-kDa protein from total T-cell lysates which became phosphorylated in the kinase assay. Phosphoamino acid analysis confirmed that this protein was phosphorylated exclusively on tyrosine residues (data not shown). Sepharose beads coupled with the nonphosphorylated form of the cytoplasmic domain of TCR ζ precipitated a number of other proteins but not the 72-kDa protein (Fig. 5C, lane 2 versus lane 3). Unconjugated beads failed to precipitate any detectable kinase activity (lane 1). These results suggest that the 72-kDa phosphoprotein binds specifically to tyrosine-phosphorylated TCR ζ.

Given the knowledge that $p56^{lck}$ and $p59^{fyn}$ are also involved in TCR signalling, it was possible that the surface TCR-associated kinase activity we detected was attributable to $p56^{lck}$ and/or $p59^{fyn}$. To address this possibility, we compared immunoprecipitates of surface TCR complexes for $p56^{lck}$ and $p59^{fyn}$ kinase activity. In Fig. 6A, the surface TCR complexes exhibit a pattern of phosphoproteins including a 72-kDa phosphoprotein which was not detected in the kinase assays of $p56^{lck}$ or $p59^{fyn}$ immunoprecipitates (lane 1 versus lanes 2 and 3). There is, however, a phosphoprotein in the TCR precipitates which migrated at a molecular weight near that of autophosphorylated $p56^{lck}$ (lane 1 versus lane 3). To examine further whether $p56^{lck}$ and/or $p59^{fyn}$ could associate and coprecipitate with surface TCR complexes following TCR engagement, anti-TCR immunoprecipitates from anti-CD3-stimulated T cells were blotted for $p56^{lck}$ and $p59^{fyn}$. Within the detection limits of the system, we were unable to detect either $p56^{lck}$ or $p59^{fyn}$ coprecipitating with surface TCR complexes. However, both proteins were readily detected in total cell lysates from 15-fold fewer cell equivalents (Fig. 6B, lanes 1 versus 2 and 4 versus 5). The band which migrated just below $p56^{lck}$ in the TCR immunoprecipitates appears to be the anti-CD3 MAb (present in lanes 1, 3, and 5) and is likely the same phosphoprotein which migrated near $p56^{lck}$ in the SDS-PAGE analysis of the kinase assays (Fig. 6A, lane 1). Lymph node

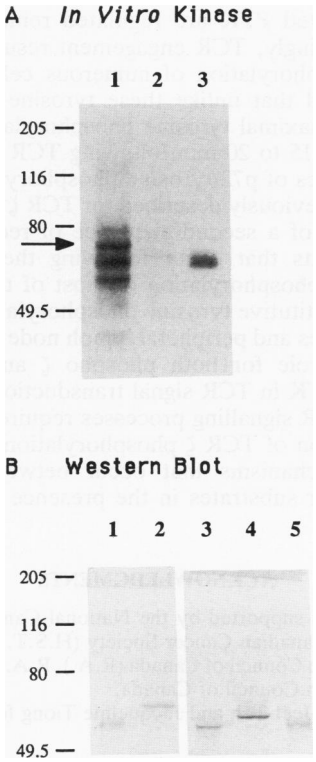


FIG. 6. Surface TCR complexes associate with a PTK distinct from p56$^{lck}$ and p59$^{fyn}$. (A) Surface TCR complexes from $3 \times 10^7$ stimulated lymph node T cells (lane 1; 10 min of 2C11 stimulation), p59$^{fyn}$ precipitates (lane 2; $5 \times 10^6$ cell equivalents), and p56$^{lck}$ precipitates (lane 3; $2 \times 10^6$ cell equivalents) were subjected to kinase assays and SDS–8% PAGE. The arrow indicates the position of the 72-kDa band. (B) Surface TCR complexes from 2C11-stimulated T cells (lanes 1 and 5; $3 \times 10^7$ cell equivalents) and total T-cell lysates (lanes 2 and 4; $2 \times 10^6$ cell equivalents) were analyzed by SDS–7.5% PAGE and immunoblotting with antibodies to p59$^{fyn}$ (lanes 1 and 2) or p56$^{lck}$ (lanes 3 to 5). Lane 3 contained 5 μg of purified 2C11. The numbers on the left are molecular sizes in kilodaltons.

T-cell lysates were also depleted of p56$^{lck}$ and p59$^{fyn}$, and the TCR-CD3 complexes were subsequently precipitated and subjected to in vitro kinase assays. Under these conditions, a 72-kDa phosphoprotein was still present despite the removal of p56$^{lck}$ or p59$^{fyn}$ (data not shown).

## DISCUSSION

TCR stimulation activates the PTK pathway, leading to the tyrosine phosphorylation of numerous cellular substrates, including the TCR ζ chain (25). In this report, we show that both lymph node T cells and thymocytes from normal C57BL/6J mice and H-Y TCR transgenic mice express a constitutively tyrosine-phosphorylated TCR ζ subunit. In H-Y TCR transgenic mice, constitutive tyrosine phosphorylation of TCR ζ can occur in the absence of the male antigen and does not require CD4 or CD8 coreceptor expression. We also demonstrate that surface TCR complexes isolated from C57BL/6J lymph node T cells contain phospho ζ and associate with 30- and 72-kDa tyrosine-phosphorylated proteins. TCR engagement of these cells leads to increased tyrosine phosphorylation of p30 and p72, as well as detection of phosphoproteins with molecular masses of 34 and 45 kDa. The surface TCR complexes also associate with a PTK which is activated upon TCR engagement. Detection of PTK activity requires the expression of phospho ζ, as cells which have been cultured for several hours exhibit a considerable loss of phospho ζ and associated PTK activity that coprecipitates with surface TCR complexes. Moreover, subsequent TCR stimulation of these lymph node T cells results in rephosphorylation of TCR ζ and reappearance of surface TCR-associated PTK activity. A tyrosine-phosphorylated cytoplasmic region of TCR ζ coupled to beads can specifically precipitate a PTK from T-cell lysates. In in vitro kinase assays, the major tyrosine-phosphorylated protein precipitated by phospho ζ-coupled beads exhibits a molecular mass of 72 kDa.

The finding in this study that the TCR ζ chain is constitutively tyrosine phosphorylated in murine lymph node T cells is in apparent contradiction to previously published reports (41, 42, 55). There are several potential explanations for these discrepant results. (i) As noted elsewhere and shown in Fig. 3, the constitutively tyrosine-phosphorylated TCR ζ chain dephosphorylates after several hours of cell culture at 37°C (30). Previous reports characterizing peripheral lymph node T cells employed purification techniques which required elevated temperatures for several hours (41, 42, 55). This may have led to the spontaneous dephosphorylation of many tyrosine-phosphorylated TCR ζ subunits. To isolate and characterize the T cells used in this study, lymph node cells were depleted of B cells at a temperature of 4°C, a condition which prevents TCR ζ dephosphorylation (32). We cannot rule out the possibility that maintaining the cells at 4°C inactivated a protein tyrosine phosphatase and/or activated a PTK, ultimately leading to TCR ζ phosphorylation (36, 42). However, this seems unlikely as dephosphorylation of the TCR ζ chain in both thymocytes and lymph node T cells requires at least 4 h of in vitro culture at 37°C (32, 50b). (ii) Earlier studies utilized $P_i$ labelling techniques to assess the status of TCR ζ phosphorylation. If the T cells used in these studies were already tyrosine phosphorylated and tyrosine phosphate turnover proceeded slowly, then little or no incorporation of $P_i$ would have occurred. (iii) We have noted some variability in the levels of tyrosine-phosphorylated TCR ζ expressed as a component of the surface-TCR complex. This may reflect a number of regulatory mechanisms that are necessary for maintenance of a constitutively tyrosine-phosphorylated TCR ζ subunit.

Expression of a constitutively tyrosine-phosphorylated surface-associated TCR ζ chain in lymph node T cells and thymocytes may have important implications for TCR signal transduction. Most studies on TCR signalling processes have used T-cell lines which do not express a constitutively tyrosine-phosphorylated TCR ζ subunit (7, 8, 36, 41, 42). In fact, the constitutive expression of phospho ζ is hypothesized to provide a negative regulatory signal preventing effective TCR signal transduction (30–32, 55). For example, previous investigations have demonstrated that immature CD4$^+$ CD8$^+$ thymocytes express a functional TCR-CD3 complex (13, 14, 33) with a constitutively tyrosine-phosphorylated TCR ζ subunit (30, 32). Following in vitro cell culturing, both the TCR number and the calcium-signalling capability of these cells are increased and the TCR ζ subunit becomes dephosphorylated (30–32). In contrast to these reports, we found no correlation between the constitutive tyrosine phosphorylation of TCR ζ and poor TCR signalling events. Thus, lymph node T cells expressing phospho ζ can respond to TCR engagement with PTK activation. Furthermore, immature CD4$^-$ CD8$^-$ thymocytes from H-Y TCR

transgenic female (50b) or male (51) mice are fully able to transduce intracellular signals despite expression of a constitutively tyrosine-phosphorylated TCR ζ chain.

The importance of phospho ζ in TCR signal transduction is underscored by the recent finding that PTK ZAP-70 specifically associates with phospho ζ following TCR stimulation, in a mechanism that likely involves either one or both SH2 domains of ZAP-70 (7, 8, 18). Tyrosine-phosphorylated TCR ζ may therefore enable recruitment of ZAP-70 to activated receptor complexes. As demonstrated in this study, lymph node T cells express a surface-associated TCR-phospho ζ complex which associates with two phosphoproteins with molecular masses of 30 and 72 kDa. Furthermore, this TCR-phospho ζ complex exhibits PTK activity. The activity of the PTK is increased following TCR engagement, and this contributes to the increased tyrosine phosphorylation of p30 and p72, as well as the detection of several additional phosphoproteins of 34, 45, and 110 kDa. An affinity ligand comprising the tyrosine-phosphorylated TCR ζ chain can precipitate a PTK, and the major tyrosine-phosphorylated protein identified in kinase assays, as determined by SDS-PAGE, exhibits an apparent molecular mass of 72 kDa. Therefore, the 72-kDa phosphoprotein identified in this system may represent the murine equivalent of ZAP-70.

To date, three PTKs have been implicated in TCR signal transduction: $p59^{fyn}$ (11, 12, 43), $p56^{lck}$ (1, 29, 39, 40, 53, 54), and ZAP-70 (7, 8, 18, 58). Recent reports have identified a 72-kDa PTK termed Syk which is involved in B-cell receptor signalling (17). Interestingly, Syk is weakly expressed in T cells (17), and it is conceivable that Syk plays a role in TCR signal transduction. In addition, although we were unable to detect $p59^{fyn}$ or $p56^{lck}$ in surface TCR precipitates in this study, it is possible that one or both kinases are involved in the regulation of TCR ζ phosphorylation and ZAP-70 kinase functions. For example, both $p59^{fyn}$ and $p56^{lck}$ have been shown to potentiate the kinase function of ZAP-70 in COS cell transfectants (8).

If the aforementioned 72-kDa protein identified in this study which coprecipitates with the surface TCR complex represents the murine equivalent of ZAP-70, one interpretation of these results is that TCR stimulation leads to activation of ZAP-70, which is already associated with the constitutively tyrosine-phosphorylated TCR ζ chain. This is consistent with the ability of $p56^{lck}$ or $p59^{fyn}$ to potentiate the phosphorylation and activation of ZAP-70 (8). However, we cannot rule out the possibility that additional ZAP-70 is recruited from the cytosol and associates with surface-associated phospho ζ following its activation by other PTKs. It is also possible that a negative regulator associates with some of the surface-associated phospho ζ and becomes displaced following TCR activation. However, we found significant PTK activity in cell surface TCR precipitates in the absence of TCR stimulation.

A recent report showed that a 70-kDa PTK, which may be ZAP-70, is capable of complexing with CD3 ε (58). Moreover, CD3 ε may function as an independent signal transduction module similar to or distinct from TCR ζ (28, 60). Given the finding that multiple CD3 components become tyrosine phosphorylated following TCR engagement (36), it is also possible that the surface TCR-phospho ζ complexes associate with a PTK via CD3 ε. Whether the 30-kDa tyrosine-phosphorylated band observed in our studies is one of the CD3 components that contribute to the binding of the 72-kDa tyrosine-phosphorylated band remains to be determined.

The mechanisms by which the *src* family PTKs and the TCR ζ-associated PTK are regulated remain to be elucidated. Interestingly, TCR engagement results in rapid and transient phosphorylation of numerous cellular substrates (15). We found that unlike these tyrosine-phosphorylated proteins, the maximal tyrosine phosphorylation of p72 was very latent, at 15 to 20 min following TCR engagement. In fact, the kinetics of p72 tyrosine phosphorylation appear to follow those previously described for TCR ζ (15). This raises the possibility of a second sequence of receptor-mediated signalling events that occur following the induction and subsequent dephosphorylation of most of the phosphoproteins. The constitutive tyrosine phosphorylation of TCR ζ in both thymocytes and peripheral lymph node T cells suggests an important role for both phospho ζ and the phospho ζ-associated PTK in TCR signal transduction. A full understanding of TCR signalling processes requires an evaluation of the regulation of TCR ζ phosphorylation, as well as the regulatory mechanisms that occur between the various PTKs and their substrates in the presence and absence of phospho ζ.

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