

A Role for the Tec Family Tyrosine Kinase Txk in T Cell Activation and Thymocyte Selection

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Summary

Recent data indicate that several members of the Tec family of protein tyrosine kinases function in antigen receptor signal transduction. Txk, a Tec family protein tyrosine kinase, is expressed in both immature and mature T cells and in mast cells. By overexpressing Txk in T cells throughout development, we found that Txk specifically augments the phospholipase C (PLC)- γ 1-mediated calcium signal transduction pathway upon T cell antigen receptor (TCR) engagement. Although Txk is structurally different from inducible T cell kinase (Itk), another Tec family member expressed in T cells, expression of the Txk transgene could partially rescue defects in positive selection and signaling in *itk*^{-/-} mice. Conversely, in the *itk*^{+/+} (wild-type) background, overexpression of Txk inhibited positive selection of TCR transgenic thymocytes, presumably due to induction of cell death. These results identify a role for Txk in TCR signal transduction, T cell development, and selection and suggest that the Tec family kinases Itk and Txk perform analogous functions.

Key words: transgenic mice • signal transduction • T cell receptors • phospholipase C • T cells

The tyrosine kinase Txk (also known as Rlk [1–3]) is a member of a new family of Src superfamily nonreceptor tyrosine kinases, the Tec family. Tec family members share Src homology 3 (SH3),¹ SH2, and tyrosine kinase catalytic domains with each other and with Src family kinases. Unlike Src family kinases such as Lck and Fyn, Tec family members do not contain a negative regulatory tyrosine in their catalytic domains nor do they contain a myristylation site in their NH₂-terminal domains. In the mouse, Tec family members include Bruton's tyrosine kinase (Btk [4]), inducible T cell kinase (Itk [5–7]), Tec (8), Txk (1, 2), and Bmx (9). All of the family members with the exception of Bmx contain a Tec homology domain, which includes a proline-rich SH3-binding domain, in their NH₂-terminal regions. All Tec family members except Txk contain a pleckstrin homology (PH) domain in their NH₂-terminal regions. In general, Src superfamily kinases acquire an "inactive" conformation when their SH2 domains interact with the negative regulatory phosphotyrosine in their catalytic domains. The Tec family kinases do not possess a neg-

ative regulatory phosphotyrosine but appear to use another negative intramolecular regulatory interaction between their SH3 and proline-rich SH3-binding domains (10).

Tec family members have restricted expression almost exclusively within hematopoietic cells. Btk is expressed in B and myeloid cell lineages (4, 11, 12), Itk in T, myeloid, and mast cell lineages (5–7), Tec in T, B, and myeloid cell lineages (8, 13, 14), Txk in T and mast cell lineages (1, 2), and Bmx in the granulocyte-monocytic lineage (9). The functions of some of these family members in the development of particular hematopoietic lineages have been investigated in human disease and in mouse model systems. Naturally occurring mutations in *btk* in humans result in X-linked agammaglobulinemia (for reviews, see references 15 and 16), revealing an absolute requirement for Btk function in B cell development in humans. In mice, mutation of *btk* results in X-linked immunodeficiency (17, 18), and *btk* knockout mice show a similar phenotype (19, 20), indicating that Btk is also important for B cell development and function in mice. T cell development and function have been analyzed in *itk* knockout mice (21–23). These mice show defects in T cell development and thymocyte selection, which are most evident when they are also transgenic for defined TCR genes (21). T cell proliferation and IL-2 production are also impaired in *itk* knockout mice in response to TCR cross-linking (21, 22).

¹Abbreviations used in this paper: B6, C57BL/6; Btk, Bruton's tyrosine kinase; DP, CD4⁺CD8⁺ double positive; DN, CD4⁻CD8⁻ double negative; Itk, inducible T cell kinase; MAP, mitogen-activated protein; PH, pleckstrin homology; PLC, phospholipase C; SH3, Src homology 3; SP, CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive.

Txk is expressed in both immature and mature T cells (1, 2); however, its role in T cell signaling and development has remained less well characterized. In a recent report, mice lacking Txk were found to exhibit only a mild impairment in TCR signaling (24). However, in the absence of both Txk and Itk, the TCR signaling defects in *itk*^{-/-} mice were exacerbated, suggesting that these proteins may perform similar functions (24). Consistent with this idea, current data indicate that both Itk and Txk as well as Btk function specifically in the signaling pathway leading to phospholipase C (PLC)- γ activation and calcium mobilization (24–27).

To study Txk function, we generated transgenic mice that overexpress Txk throughout T cell development. Although T cell maturation appeared grossly unaffected, thymocytes and peripheral T cells from *txk* transgenic mice were hyperresponsive to TCR stimulation as assessed by increased PLC- γ 1 phosphorylation and enhanced calcium flux. Txk overexpression also resulted in enhanced IL-2 production after TCR engagement. Txk overexpression inhibited positive selection in TCR transgenic mice, but partially rescued the defect in positive selection in TCR transgenic, *itk*^{-/-} mice. Since overexpression of Txk augments the TCR signaling response, the inhibition of positive selection in *itk*^{+/+} *txk* transgenic mice may be due to the induction of programmed cell death in developing thymocytes. Collectively, these results identify a role for Txk in TCR signal transduction, T cell development, and thymocyte selection, and provide evidence that Txk and Itk perform analogous functions in T cells.

Materials and Methods

Mice. For construction of the Txk transgenic vector, most of the 3' untranslated *txk* cDNA sequence was first eliminated by digestion of the Bluescript SK plasmid containing the *txk* cDNA (1) with BspM1 and XhoI and resealing of the plasmid incorporating a SalI linker to create a SalI site at the 3' end of the *txk* cDNA. Then the EcoRI-SalI *txk* cDNA fragment was cloned into a plasmid containing the CD2 promoter and enhancer (28) and sequences from TCR- ζ genomic DNA (29). The CD2/Txk/TCR- ζ sequences were separated from vector sequences by digestion with NotI and were isolated for pronuclear injection as described previously (29). Txk transgenic mice were backcrossed to C57BL/6 (B6) mice for >10 generations. TCR transgenic mice used in these studies included H-Y and AND TCR transgenic mice. H-Y mice express an MHC class I-restricted TCR for the male antigen H-Y (30), and AND mice express an MHC class II-restricted TCR specific for pigeon cytochrome C (31). All TCR transgenic mice were in the H-2D^b background. *itk* knockout mice were kindly provided by D. Littman (New York University Medical Center, New York, NY).

Cell Culture. Thymocytes were cultured when necessary in RPMI complete medium as described previously (1). The resting period for thymocytes (see Fig. 4) was 5 h at 37°C, 5% CO₂.

Antibodies. Polyclonal antiserum against Txk peptide 1 (CKPLPPLPQEPEDER), found near the NH₂ terminus of Txk, conjugated to KLH was raised in rabbits by Covance, Inc. Peptide synthesis was performed by Biosynthesis, Inc. Anti-CD3 (145-2C11) and anti-CD28 (37.51) were prepared in our labora-

tory by protein G column purification (Sigma Chemical Co.). Polyclonal anti-Lck serum and anti-ZAP-70 ascites were provided by L. Samelson (National Institutes of Health). T3.70-FITC was prepared in our laboratory. Commercial antibodies used include anti-CD3-FITC (PharMingen), anti-CD4-PE (PharMingen), anti-CD8a-Quantum red (Sigma Chemical Co.), anti-V α 11-FITC (PharMingen), H57-597-biotin (PharMingen), polyclonal anti-bovine PLC- γ 1 (Upstate Biotechnology), mixed monoclonal anti-bovine PLC- γ 1 (Upstate Biotechnology), and antiphosphotyrosine (4G10; Upstate Biotechnology).

Metabolic Labeling and Immunoprecipitation. 10⁸ thymocytes were incubated for 30 min at 37°C in 5 ml complete medium containing 5 mCi TRAN³⁵SLABEL (ICN Biomedicals). For immunoprecipitation, cell lysates were prepared by incubating cells in NP-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM Hepes, 1 mM EDTA, 1 mM Na₃VO₄, 1% NP-40, and 1× CompleteTM protease inhibitor cocktail; Roche) for 30 min on ice. Lysates were cleared by microcentrifugation for 15 min at 12,000 *g*. After incubation of lysates with appropriate antibodies, complexes were collected with protein A-Sepharose (Sigma Chemical Co.) and washed three times with NP-40 wash buffer (same as NP-40 lysis buffer except with 0.2% NP-40). Samples were boiled in reducing sample buffer (0.175 M Tris, pH 6.8, 30% glycerol, 3% SDS, 15% β -mercaptoethanol, 0.1% bromophenol blue) and subjected to PAGE. 10% polyacrylamide gels were used in all cases except for immunoprecipitation with the ZAP-70 antibody, in which case 12% gels were used. In the case of metabolic labeling, gels were treated with DMSO and 20% 2,5-diphenylloxazole (PPO) in DMSO, dried, and exposed to BioMax MR film (Eastman Kodak Co.) and a BioMax TranScreen LE intensifying screen. In cases of Western blotting, gels were transferred by standard methods (32). For in vitro TCR cross-linking (see Fig. 4), 10⁸ thymocytes were resuspended in RPMI 1640 at 10⁷ cells/ml. 100 μ g of biotinylated H57-597 and 100 μ g of biotinylated GK1.5 (both prepared in our laboratory) were added for 10 min on ice. After centrifugation, the cells were resuspended in RPMI 1640 containing 20 μ g/ml streptavidin (Life Technologies) for various times at 37°C. Extracts were then prepared in Triton X-100 lysis buffer (same as NP-40 lysis buffer except with 1% Triton X-100 instead of 1% NP-40) as described above.

In Vitro Kinase Assay. After immunoprecipitation with anti-Txk antiserum or anti-Txk antiserum with Txk peptide 1 and washing with NP-40 wash buffer minus EDTA, complexes were incubated with kinase buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MnCl₂, 5 mM MgCl₂, 2 μ M NaATP, 10 μ M [γ -³²P]ATP, and 10 μ g enolase) for 15 min at room temperature. Complexes were then boiled in reducing sample buffer and subjected to electrophoresis on 10% polyacrylamide gels. The gels were then fixed, Coomassie blue stained (32), and exposed to x-ray film (X-OMAT AR; Eastman Kodak Co.).

Cytometry and Measurements of Calcium Flux. Standard flow cytometry was performed as described previously (33) using a Becton Dickinson FACScanTM and CELLQuestTM software. For calcium flux studies, cells were washed and resuspended in HBSS/FBS (HBSS with calcium and magnesium, 10 mM Hepes, and 1% FBS). The calcium probe indo-1 and the detergent Pluronic (Molecular Probes) were added at final concentrations of 10 μ M and 300 μ g/ μ l, respectively. After washing, the cells were also stained with anti-CD8 α -FITC (PharMingen) and anti-CD4-PE (PharMingen). Cells were analyzed on a FACS VantageTM (Becton Dickinson) equipped with an argon laser tuned to 488 nm and a krypton laser tuned to 360 nm. Indo-1 fluorescence was analyzed at 390/20 and 530/20 for bound and free probe, respec-

tively. The signals for bound and unbound indo-1 were collected in linear mode. The FACS Vantage™ was equipped with two modifications necessary for reproducible measurement of TCR-induced calcium flux: a Time Zero injection module (Cytex) and ratio offset, so that the gain of the fluorescence ratio could be increased while keeping the baseline signal in the lower channels. For each stimulation, an aliquot of cells was warmed at 37°C for 3 min before stimulation with TCR cross-linking antibody. Data were collected for 30 s, at which time varying amounts of biotinylated H57-597 (0.2–10 µg; PharMingen) were added in a total volume of 50 µl. At 60 s, 20 µg of streptavidin (Life Technologies), also in a total volume of 50 µl, was injected. Cells were collected at a rate of 1,000–2,000 cells/s.

The kinetic data shown in Figs. 3 and 8 were generated using the software programs WinList (Verity Software House, Inc.) and Canvas (Deneba Software). The percentage of cells that responded by an increase in intracellular calcium after stimulation with TCR cross-linking antibody was determined using Multitime Software for Analysis of Kinetic Flow Cytometry Data (Phoenix Flow Systems). A threshold of fluorescence ratio was determined in the window of time before antibody addition. This threshold was then applied to the time window after the addition of antibody and streptavidin. The maximum number of cells above the threshold averaged over a 6-s time interval was expressed as a percentage of all of the cells in the same interval. From this number, we subtracted the percentage of cells above threshold in the resting time window to obtain the percentage of cells responding.

Cytokine ELISA Measurements. CD4⁺ cells were isolated from total LN cells using positive selection magnetic cell sorting

(MACS; Miltenyi Biotec) according to the manufacturer's recommendations. CD4⁺ cell populations were >95% pure as measured by standard flow cytometry. Measurements for levels of IL-2, IL-4, and IFN-γ were performed by ELISA using the reagents and protocol from PharMingen.

Results

Generation of *txk* Transgenic Mice. To examine the role of Txk in T cell development and TCR signal transduction, we overexpressed Txk in transgenic mice. Transgene expression was driven by the human CD2 promoter/enhancer (diagrammed in Fig. 1 a). The CD2 promoter was chosen to drive Txk expression because it directs expression early and throughout T cell development, i.e., in CD4⁻CD8⁻ (double negative [DN]), CD4⁺CD8⁺ (double positive [DP]), and CD4⁺CD8⁻ and CD4⁻CD8⁺ (single positive [SP]) thymocytes and in mature peripheral T cells (29; and Love, P.E., unpublished observations), approximating the expression pattern of endogenous *txk* (1). Five founder lines were established, and two founder lines showing the highest levels of expression were analyzed. Fig. 1 shows Txk protein overexpression in the founder line showing highest expression. Extracts from hemizygous and homozygous transgenic thymocytes were immunoprecipitated with anti-Txk antibody after metabolic labeling (Fig. 1 b). In vitro immune complex kinase assays were also performed using anti-Txk

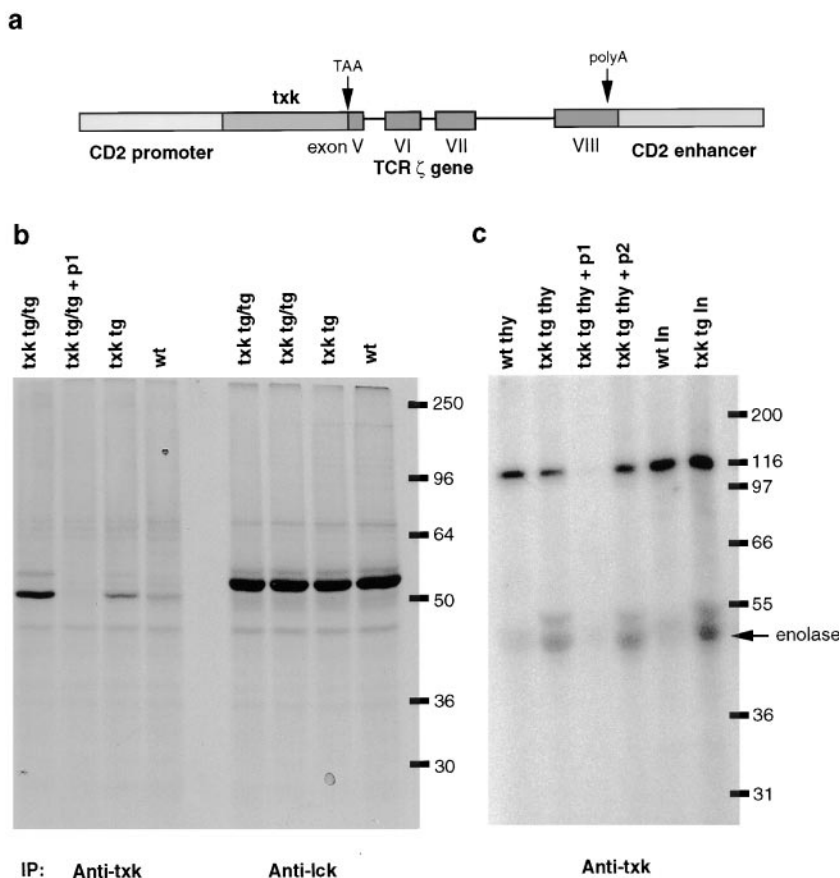


Figure 1. Txk overexpression in transgenic mice. (a) Transgenic construct for expressing Txk. *txk* cDNA expression is driven by the human CD2 promoter and enhancer. Sequences from TCR-ζ including intronic sequences were included to enhance message stability. (b) Metabolic labeling and immunoprecipitation. Thymocytes from *txk* homozygous (*txk tg/tg*), *txk* hemizygous (*txk tg*), or nontransgenic (*wt*) mice were subjected to metabolic labeling using [³⁵S]methionine and cysteine before immunoprecipitation (IP) with anti-Txk or anti-Lck polyclonal antiserum. Anti-Lck was used to show equivalent loading. In lane 2, an excess of the peptide used for immunization in preparing the anti-Txk antiserum (p1) was included in the immunoprecipitation. Molecular mass markers (in kD) are shown at the right. (c) In vitro kinase assay. In lanes 1–4, extracts from thymocytes from wild-type and *txk* hemizygous transgenic mice were immunoprecipitated with anti-Txk antiserum followed by incubation with [³²P]ATP and the substrate enolase to assess kinase activity. Increased levels of phosphorylated enolase are seen in the Txk transgenic thymocytes. This phosphorylation is blocked by peptide 1 (p1, used for immunization in preparing the Txk antiserum) but not by another peptide from Txk (p2). Extracts from LNs (ln) were also analyzed. The identity of the high molecular mass phosphorylated peptide (~116 kD) is unknown.

antiserum to show the functionality of Txk in Txk transgenic mice. As shown in Fig. 1 c, the tyrosine kinase substrate enolase was phosphorylated to a greater extent in extracts immunoprecipitated from *txk* transgenic thymocytes compared with extracts from wild-type thymocytes. Greater enolase phosphorylation was also seen for *txk* transgenic LNs, as was expected from the expression pattern of the CD2 promoter (28). Data averaged from several experiments show that Txk protein overexpression was ~10-fold in *txk* homozygous transgenic mice. Increases in in vitro kinase activity were also ~10-fold in *txk* homozygous transgenic mice.

Phenotype of *txk* Transgenic Mice. *txk* transgenic mice exhibited normal development and fertility. T cell maturation appeared unaffected as assessed by normal numbers of thymocytes and T lymphocytes. In addition, percentages of thymocyte subsets in *txk* transgenic mice (DN, DP, and SP thymocytes) were normal, as were the ratios of CD4⁺ to CD8⁺ cells in thymus and in LNs as determined from several experiments (Fig. 2, and data not shown). Staining for CD3 (Fig. 2) and the following markers (data not shown) showed similar profiles for *txk* transgenic and nontransgenic thymocytes (TCR- α/β , TCR- γ/δ , CD5, CD69, and CD24) and lymphocytes (TCR- α/β , TCR- γ/δ , CD5, CD44, and CD62L).

Augmentation of TCR-mediated Signaling in *txk* Transgenic Mice. Because the Tec family kinases Itk and Btk have been shown to regulate calcium flux in T and B cells, respectively (26, 27), we analyzed calcium mobilization in response to TCR cross-linking in *txk* transgenic thymocytes and lymphocytes. Cells were preloaded with the calcium dye indo-1, stained for CD4 and CD8, and then stimulated with biotinylated anti-TCR- β antibody. At low concentrations of anti-TCR- β (1.0 $\mu\text{g/ml}$), the calcium response was enhanced in *txk* transgenic DP thymocytes relative to nontransgenic DP thymocytes as assessed by: (a) the percentage of cells responding (18.6 vs. 3.2%, respectively); (b) the accelerated kinetics of the early calcium response; and (c) the increased amplitude of the kinetic response (Fig. 3 a). However, with higher concentrations of anti-TCR- β (10 $\mu\text{g/ml}$), the calcium response was equivalent in *txk* trans-

genic and nontransgenic thymocytes (data not shown). In addition, the kinetics of the delayed calcium response were similar in *txk* transgenic and nontransgenic mice (Fig. 3). SP thymocytes and LN T cells from *txk* transgenic mice exhibited similarly enhanced responses compared with equivalent populations of nontransgenic cells when stimulated with submaximal concentrations of antibody (Fig. 3). Again, in *txk* transgenic mice the percentage of CD4 SP cells responding was increased (75.3 vs. 37.6% cells responding for CD4⁺ thymocytes, and 41.0 vs. 33.2% cells responding for CD4⁺ lymphocytes), as were the kinetics and amplitude of the early calcium response (Fig. 3).

One outcome of an enhanced TCR-mediated calcium response should be increased IL-2 production. We measured cytokine production in CD4⁺ LN cells from *txk* transgenic mice after stimulation by plate-bound anti-CD3 or anti-CD3 plus anti-CD28 (Table I). CD4⁺ LN cells from *txk* transgenic mice produced significantly more IL-2 than nontransgenic CD4⁺ T cells. Moreover, the effect of Txk overexpression was dose dependent, since lymphocytes from *txk* homozygous transgenic mice produced more IL-2 than lymphocytes from *txk* hemizygous transgenic mice. However, no enhancement in proliferation was observed either in lymphocytes or thymocytes from *txk* transgenic mice in response to TCR cross-linking using similar conditions to those used for analysis of cytokine production (data not shown).

It has been previously reported that *txk* is preferentially expressed in Th1 versus Th2 T cell clones (2). To investigate whether Txk overexpression would direct CD4⁺ lymphocytes down either a Th1 or Th2 developmental pathway, we also measured levels of IL-4 and IFN- γ in response to TCR cross-linking. Table I shows that both IL-4 and IFN- γ secretion were increased in *txk* transgenic CD4⁺ LN cells, indicating an overall increase in cytokine production in *txk* transgenic mice but no bias toward either the Th1- or Th2-specific cytokine profile under these conditions.

Txk Selectively Enhances the Calcium Signaling Pathway. To further investigate where in the TCR-mediated signal transduction pathway Txk exerts its effect, we next examined phosphorylation levels and activities of several signal transduction molecules known to be activated upon TCR

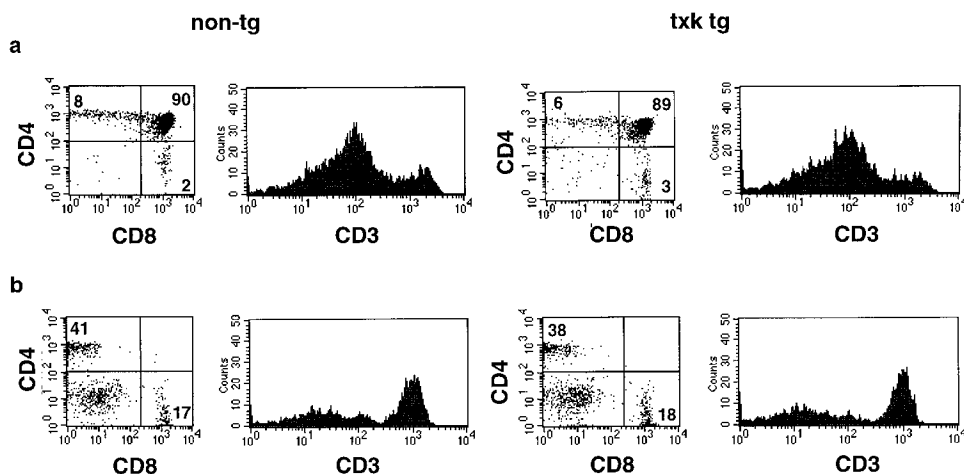


Figure 2. Phenotype of *txk* transgenic mice. (a) Thymocytes from *txk* transgenic (*txk* tg) and nontransgenic (non-tg) mice were stained with anti-CD4, anti-CD8, and anti-CD3 and analyzed by flow cytometry. CD4 vs. CD8 profiles are shown with percentages of thymocytes indicated within quadrants of the dot plots. Histograms of anti-CD3 staining are also shown. (b) LN cells from *txk* transgenic and nontransgenic mice were stained with anti-CD4, anti-CD8, and anti-CD3 and analyzed by flow cytometry as described in a.

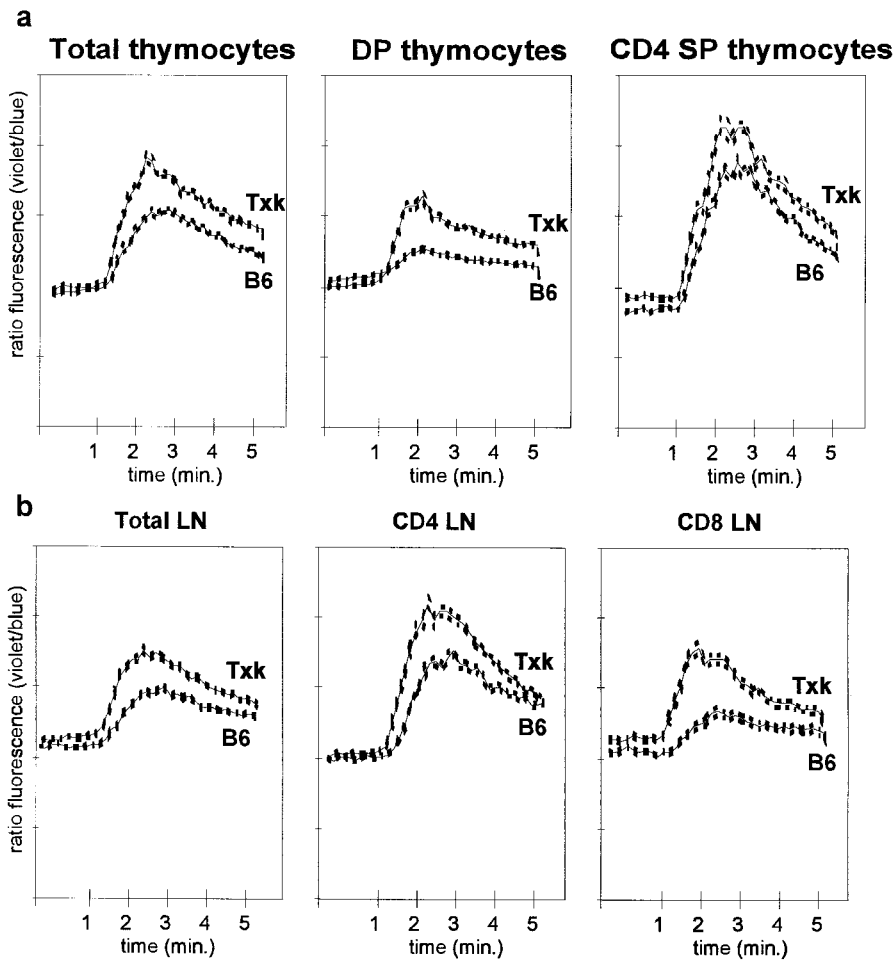


Figure 3. The calcium response is enhanced in Txk transgenic thymocytes and lymphocytes. (a) Wild-type B6 and Txk transgenic thymocytes were preloaded with the calcium dye indo-1 and then stimulated by the addition of 1.0 μg of biotin-H57 (anti-TCR- β) antibody at 30 s and 20 μg of streptavidin (cross-linking agent) at 60 s. Fluorescence indicating bound and unbound indo-1 was monitored over time (5 min), and kinetic data were analyzed using Win-List software. The cells were also stained with anti-CD4 and anti-CD8, so that calcium flux data could be obtained on gated thymocyte subpopulations. (b) B6 and Txk transgenic LN cells were treated and analyzed as described in a, except that 0.5 μg of biotin-H57 was used. Results are representative of at least four separate experiments.

Table I. Increased Cytokine Production by Txk Transgenic CD4⁺ Lymphocytes

Cell type	Treatment	IL-2	IL-4	IFN- γ
		pg/ml	pg/ml	pg/ml
wt	Control	ND	ND	ND
txk tg	Control	ND	ND	ND
txk tg/tg	Control	ND	ND	ND
wt	Anti-CD3	ND	8	1,134
txk tg	Anti-CD3	12	52	7,193
txk tg/tg	Anti-CD3	56	65	8,245
wt	Anti-CD3 plus anti-CD28	24	34	19,258
txk tg	Anti-CD3 plus anti-CD28	2,994	120	43,780
txk tg/tg	Anti-CD3 plus anti-CD28	6,064	146	26,435

IL-2, IL-4, and IFN- γ production were determined by ELISA from CD4⁺ lymphocytes from wild-type (wt), *txk* homozygous transgenic (tg/tg), and *txk* hemizygous transgenic (tg) mice. Lymphocytes were plated under control conditions or with plate-bound anti-CD3 or plate-bound anti-CD3 and anti-CD28. Supernatants were analyzed after 24 (IL-4) or 48 (IL-2, IFN- γ) h. The results are representative of three experiments. ND, none detected.

engagement. Early events after engagement of the TCR are phosphorylation of the ζ chain and CD3 subunits of the TCR by Lck, and recruitment of ZAP-70 to the TCR complex. Recruitment of adapter molecules to the TCR complex then results in activation of two more distal pathways of signal transduction. The first pathway involves PLC- γ 1-dependent phosphoinositide lipid metabolism and leads to the release of calcium from intracellular stores and the activation of protein kinase C. Another major signal transduction pathway involves activation of Ras and the mitogen-activated protein (MAP) kinases (for a review, see reference 34). Tyrosine phosphorylation levels of early effectors of TCR signal transduction (TCR- ζ , ZAP-70, Lck) did not vary between *txk* transgenic and B6 control thymocytes either before or after TCR engagement (Fig. 4, and data not shown). Similarly, tyrosine phosphorylation (Vav) or kinase activity (Erk-2) of signal transduction molecules in the MAP kinase pathway was identical in *txk* transgenic and nontransgenic thymocytes (data not shown). Notably, tyrosine phosphorylation of PLC- γ 1 was reproducibly greater in ex vivo thymocytes from *txk* transgenic mice than from nontransgenic thymocytes (Fig. 4; these results are representative of seven separate experiments). Since ex vivo thymocytes are known to be activated to

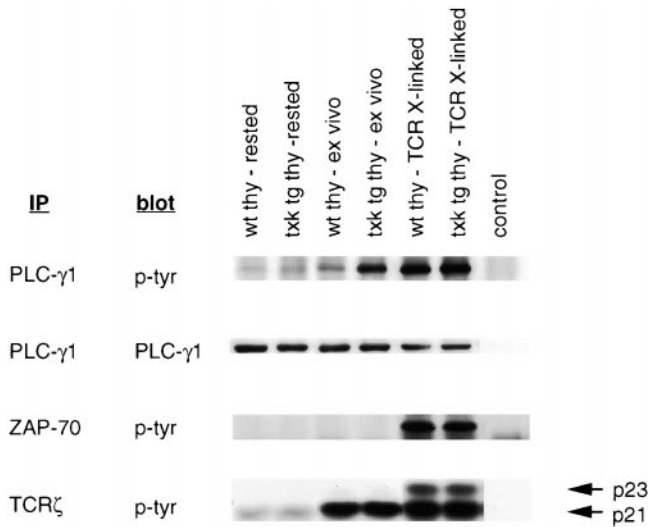


Figure 4. Enhanced phosphorylation of PLC- γ 1 in *txk* transgenic thymocytes. Extracts from wild-type B6 (wt thy) or *txk* transgenic thymocytes (*txk* tg thy) were made immediately after removal from the animal (ex vivo), after a period of incubation of 5 h at 37°C (rested), or after treatment with biotinylated anti-TCR (H57) and anti-CD4 (GK1.5) and cross-linking with streptavidin for 5 min. The control lane represents immunoprecipitation of lysis buffer instead of cell extract. Immunoprecipitations with anti-PLC- γ 1 (top two rows) or anti-ZAP-70 (bottom two rows) were performed. In the first row, the PLC- γ 1 immunoprecipitation (IP) was followed with an antiphosphotyrosine (4G10) blot. The blot was stripped and reprobed with an additional PLC- γ 1 antibody (second row). In the third and fourth rows, the ZAP-70 immunoprecipitation was followed by antiphosphotyrosine (p-tyr) blotting. The region of the blot showing ZAP-70 (70 kD) is shown in the third row, and the region showing TCR- ζ (p21 and p23 forms) is shown in the last row.

some extent due to contact with thymic epithelial cells (35), we also determined the tyrosine phosphorylation levels of PLC- γ 1 in thymocytes that had been “rested” by incubation at 37°C for 5 h in the absence of thymic epithelial cells. In rested thymocytes, the level of PLC- γ 1 phosphorylation was low and appeared equivalent in control and *txk* transgenic thymocytes (Fig. 4). In addition, PLC- γ 1 phosphorylation was equivalent in thymocytes from *txk* transgenic and nontransgenic mice that had been activated for 5 min with anti-TCR plus anti-CD4 antibodies (Fig. 4). However, a time course analysis revealed that at early time points after TCR engagement (0.5–1 min), PLC- γ 1 phosphorylation was greater in thymocytes from *txk* transgenic mice (Fig. 5). This effect was transgene dose dependent, as the level of PLC- γ 1 phosphorylation was greater in homozygous transgenic than in hemizygous transgenic mice (Fig. 5). Interestingly, at later time points (>1 min) the extent of PLC- γ 1 phosphorylation was equivalent in nontransgenic, hemizygous transgenic, and homozygous transgenic mice (Fig. 5). The amount of inositol trisphosphate generated in thymocytes from nontransgenic, hemizygous transgenic, and homozygous transgenic mice was also equivalent at 1, 2, and 5 min after TCR cross-linking (data not shown); however, inositol trisphosphate levels at earlier (<1 min) time points were not measured. Together these results reveal that within a certain window, *txk* transgenic T cells appear

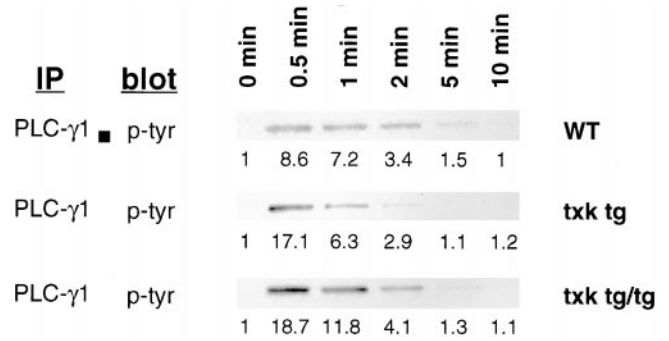


Figure 5. Time course of PLC- γ 1 phosphorylation after TCR cross-linking. Thymocytes from nontransgenic (WT), *txk* hemizygous transgenic (*txk* tg), and *txk* homozygous transgenic (*txk* tg/tg) mice were isolated, enumerated, and rested at 37°C for 5 h. Rested thymocytes (10^8) were then incubated with biotinylated anti-TCR (H57) and anti-CD4 (GK1.5) on ice and cross-linked with streptavidin at 37°C for the times indicated. Cell lysates were immunoprecipitated (IP) with anti-PLC- γ 1, resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with antiphosphotyrosine (p-tyr). The blot was subsequently stripped and reprobed with PLC- γ 1 antibody. Numbers indicate relative band intensity of the antiphosphotyrosine blot as determined by densitometric scanning and averaging to the relative intensity of bands obtained by PLC- γ 1 reblotting.

hyperactivated as measured by enhanced calcium flux kinetics and by the level of PLC- γ 1 tyrosine phosphorylation.

Effect of *Txk* Overexpression on Thymocyte Selection. A sensitive measure of the effects of T cell activation on thymocyte development can be obtained using TCR transgenic mice to examine thymocyte positive and negative selection. To determine if positive selection was affected in *txk* transgenic mice, we used the H-Y transgenic TCR (30) and the AND transgenic TCR (31). The H-Y transgenic TCR is class I restricted and H-2D^b specific. The antigen for the H-Y transgenic TCR is from the male-specific protein H-Y, and T cells expressing the transgenic TCR undergo positive selection in female mice and negative selection in male mice. In female H-Y transgenic mice, both the percentage and total number of CD8 SP thymocytes expressing high levels of the transgenic TCR (as detected by staining with the H-Y TCR clonotype-specific antibody T3.70) were reduced in *txk* transgenic mice compared with non-*txk* transgenic littermates (Fig. 6). This effect was observed consistently within multiple individual experiments comparing age-matched mice (Fig. 6 b). Moreover, in all experiments, the percentage and total number of transgenic TCR^{hi} (T3.70^{hi}) DP thymocytes (the direct precursors of T3.70^{high} CD8 SP thymocytes) were increased in *txk* transgenic mice (Fig. 6). Again, the effect of the *txk* transgene was dose dependent, since the reduction in T3.70^{hi} CD8 SP thymocytes was greater in homozygous *txk* transgenic mice than in hemizygous *txk* transgenic mice (Table II). Thus, overexpression of *Txk* inhibited thymocyte positive selection in H-Y females, resulting in a specific reduction in the number of T3.70^{hi} CD8 SP thymocytes.

Similar results were obtained in mice expressing the class II-restricted transgenic TCR, AND. The number of transgenic TCR^{hi} CD4 SP thymocytes (as detected by staining

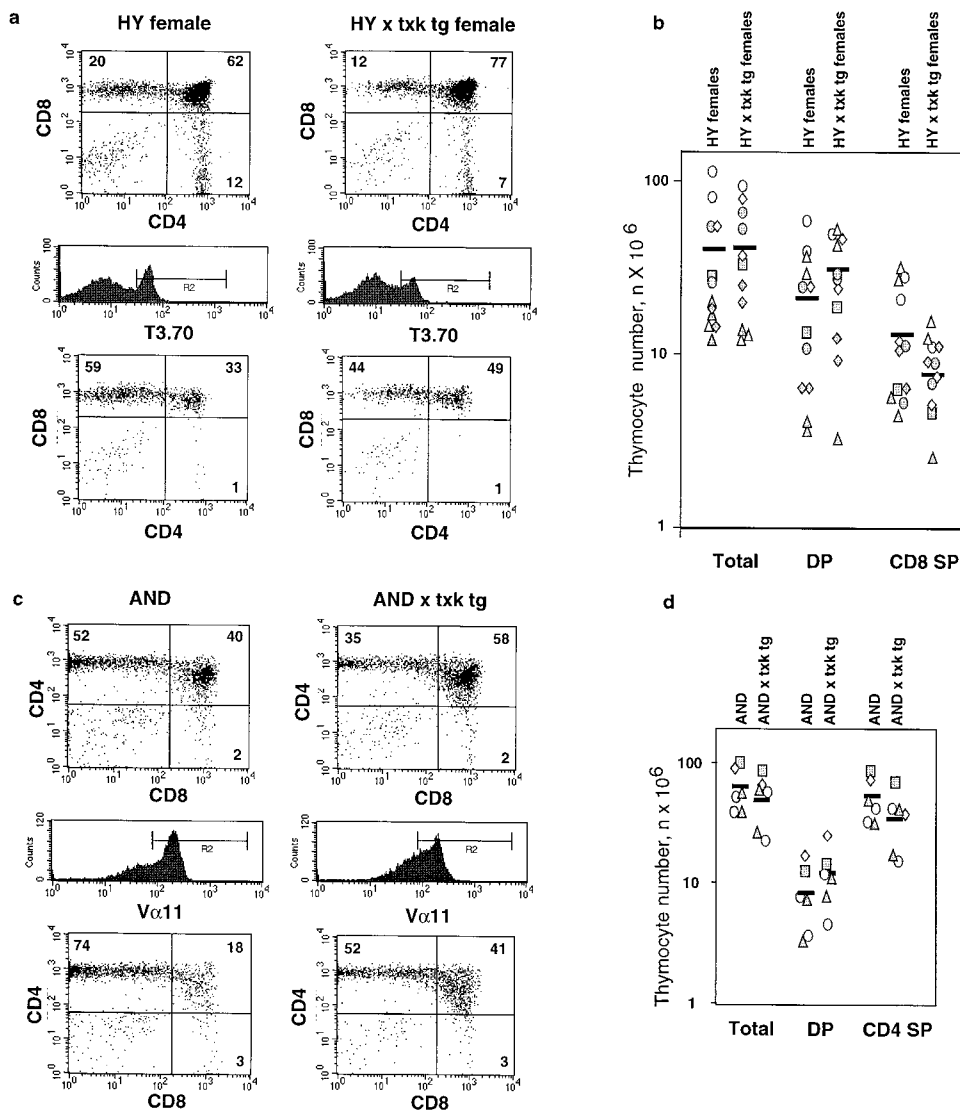


Table II. Dosage Effect of *Ttk* Transgene in H-Y TCR Transgenic Female Mice

		No. DP T3.70 ^{high} thymocytes	No. CD8 SP T3.70 ^{high} thymocytes
		$\times 10^6$	$\times 10^6$
Experiment 1	ttx tg	7.56	4.71
	ttx tg/tg	8.58	2.20
Experiment 2	ttx tg	4.69	5.53
	ttx tg/tg	6.04	3.83

Thymocytes from littermate H-Y \times *ttx* hemizygous transgenic female mice (*ttx* tg) and H-Y \times *ttx* homozygous female mice (*ttx* tg/tg) were stained with anti-CD4, anti-CD8, and T3.70 antibodies and analyzed by flow cytometry. Numbers of T3.70^{high} DP and T3.70^{high} CD8 SP thymocytes were determined.

Figure 6. Positive selection in *Ttk* transgenic mice. (a) Positive selection in *ttx* transgenic (tg) \times H-Y TCR transgenic female mice. Thymocytes from representative H-Y female and H-Y \times *ttx* transgenic littermate female mice were stained with anti-CD4, anti-CD8, and T3.70 (which stains the H-Y transgenic TCR) antibodies and analyzed by flow cytometry. CD4 vs. CD8 profiles of total (top panel) and T3.70^{hi} (gated) thymocytes (bottom panel) are shown. Percentages of thymocytes are shown within the dot plots. Single color histograms of T3.70 staining are also shown to indicate placement of the gates. (b) Thymocyte numbers in H-Y female and H-Y \times *Ttk* transgenic littermate female mice. Numbers of total thymocytes, DP thymocytes, and CD8 SP thymocytes (gated as T3.70^{hi}) for $n = 11$ mice are shown. Identical symbols indicate individual experiments in which age-matched mice were chosen. (c) Positive selection in *ttx* \times AND TCR littermate transgenic mice. Thymocytes from representative AND and *ttx* \times AND transgenic mice were stained with anti-CD4, anti-CD8, and anti-V α 11 (which recognizes the TCR- α chain of the AND transgenic TCR) antibodies and analyzed by flow cytometry. CD4 vs. CD8 profiles of total (top panel) and V α 11^{hi} (gated) thymocytes (bottom panel) are shown. Single color histograms of V α 11 staining are also shown to indicate placement of the gates. (d) Thymocyte numbers in AND and *ttx* \times AND littermate transgenic mice. Numbers of total thymocytes, DP, and CD4 SP thymocytes (gated as V α 11^{hi}) for $n = 6$ mice. Identical symbols indicate individual experiments in which age-matched mice were chosen.

with antibody specific for V α 11) was decreased in *ttx* \times AND mice relative to non-*ttx* transgenic AND littermates (Fig. 6, c and d). As with the H-Y transgenics, total thymocyte numbers were similar between AND and *ttx* \times AND mice, and the number of transgenic TCR^{hi} (V α 11^{hi}) DP thymocytes was increased in *ttx* \times AND relative to AND mice (Fig. 6, c and d). The decrease in SP transgenic TCR^{hi} thymocytes in *ttx* \times H-Y and *ttx* \times AND mice suggests that either fewer DP transgenic TCR^{hi} thymocytes are positively selected in these mice, or that the transgenic TCR^{hi} SP thymocytes normally generated by positive selection are undergoing negative selection.

Overexpression of Ttk Can Partially Rescue the Developmental Defect in itk^{-/-} Mice. Since *Ttk* and *Itk* are related kinases that appear to have similar functions in the calcium signaling pathway, we also examined whether *Ttk* could substitute for *Itk*. The efficiency of positive selection is markedly reduced in *itk^{-/-}* mice as measured in both H-Y TCR

transgenic and AND TCR transgenic mice (21; and Fig. 7). Significantly, introduction of the *txk* transgene into the *itk*^{-/-} background partially restored the generation of transgenic TCR^{hi} SP thymocytes (Fig. 6) and peripheral T cells (data not shown) in both H-Y TCR and AND TCR transgenic mice. Moreover, the unusual transgenic TCR^{hi} population of DN thymocytes, which is expanded in *itk*^{-/-} TCR transgenic mice (21), was reduced to levels comparable to those seen in *itk*^{+/-} or *itk*^{+/+} mice upon expression of the *txk* transgene (Fig. 7). In addition, both the TCR-induced calcium response (Fig. 8 a) and TCR-induced PLC- γ 1 tyrosine phosphorylation (Fig. 8 b) were normalized in *itk*^{-/-} *txk* transgenic mice. These results demonstrate that, in spite of the structural differences between Txk and Itk, overexpression of Txk can at least partially reverse the developmental and signal transduction defects in *itk*^{-/-} mice.

Discussion

By overexpressing the Tec family kinase Txk in transgenic mice, we observed specific effects on TCR signaling, which include selective augmentation of the calcium signaling pathway, enhanced cytokine production, and alterations in positive selection. These effects were not due to a chance integration effect of the transgene, since a second founder line with lower Txk expression had similar but reduced effects (data not shown).

Engagement of the TCR results in the activation of at least two major intracellular signaling pathways (for a review, see reference 34). One pathway leads to the activation of Ras and MAP kinases, the other to the initiation of phosphoinositol lipid metabolism. Activation of phosphatidylinositol 3'-hydroxyl kinase results in the generation of phosphatidylinositol 3,4-bisphosphate in addition to other

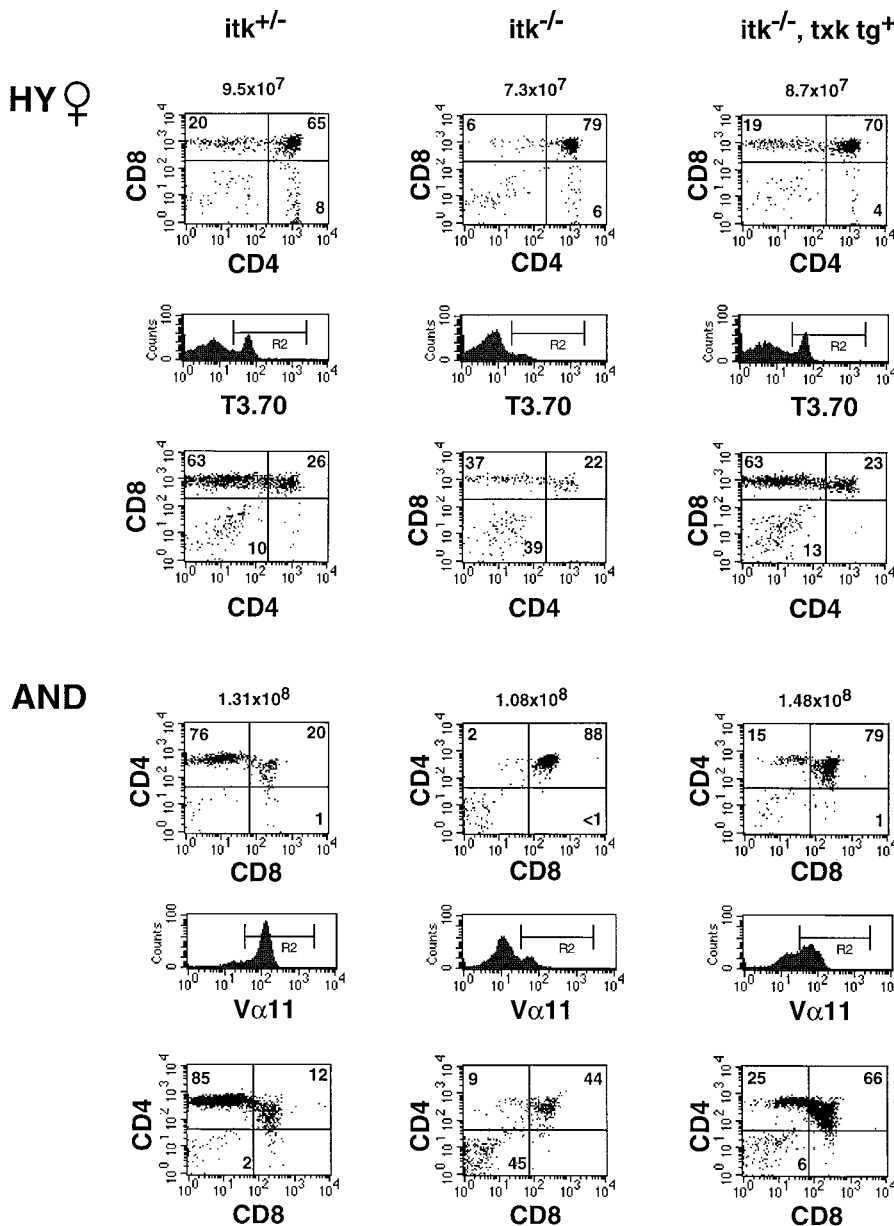


Figure 7. Txk reconstitution of *itk*^{-/-} mice. Positive selection was assessed in TCR transgenic *itk*^{+/-} or *itk*^{-/-} thymocytes in the presence or absence of the *txk* transgene. Thymocytes from H-Y TCR transgenic female and AND TCR transgenic mice were stained with anti-CD4, anti-CD8, and T3.70 (which stains the H-Y transgenic TCR) or anti-V α 11 (which stains the AND transgenic TCR) and analyzed by flow cytometry. CD4 vs. CD8 profiles of total (top panels in each set) or T3.70^{hi} (gated) or V α 11^{hi} (gated) thymocytes (bottom panels in each set) are shown. Percentages of thymocytes are shown within the dot plots. Histograms of T3.70 and V α 11 staining on total thymocytes are shown below the dot plots to indicate placement of the gates. Numbers above the plots indicate the total number of thymocytes. The results shown are representative of at least three experiments.

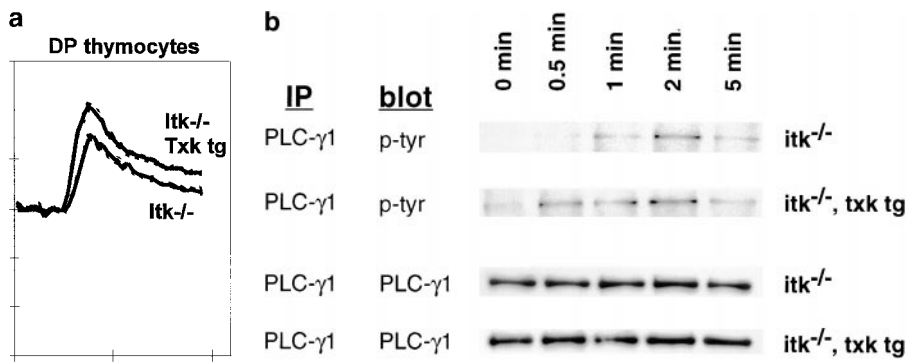


Figure 8. (a) The calcium response is enhanced in *Itk*^{-/-} *txk* transgenic thymocytes. Thymocytes from *Itk*^{-/-} and *Itk*^{-/-} *txk* transgenic mice were preloaded with the calcium dye indo-1 and then stimulated by the addition of 5.0 μg of biotin-H57 (anti-TCR-β) antibody at 30 s and 20 μg of streptavidin (cross-linking agent) at 60 s. Fluorescence indicating bound and unbound indo-1 was monitored over time (5 min), and kinetic data were analyzed using WinList software. The cells were also stained with anti-CD4 and anti-CD8, so that calcium flux data could be obtained on gated DP thymocytes. Ratio fluorescence (violet/blue, y axis) is plotted against time (x axis). (b) Time course of

PLC-γ1 phosphorylation after TCR cross-linking. Thymocytes from *Itk*^{-/-} or *Itk*^{-/-} *txk* hemizygous transgenic (*Itk*^{-/-}, *txk* tg) mice were isolated and enumerated. Thymocytes (10⁸) were then incubated with biotinylated anti-TCR (H57) and anti-CD4 (GK1.5) on ice and cross-linked with streptavidin at 37°C for the times indicated. Cell lysates were immunoprecipitated with anti-PLC-γ1, resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with antiphosphotyrosine (p-tyr). The blot was subsequently stripped and reprobed with PLC-γ1 antibody to assess loading consistency.

phosphoinositol lipids. The products of phosphatidylinositol 3,4-bisphosphate breakdown by PLC-γ stimulate calcium release from intracellular stores (36) and activate the protein kinase C pathway (34). Several Tec family members have been implicated in phosphoinositol lipid metabolism pathways. Phosphatidylinositol 3'-hydroxyl kinase has been shown to activate the kinase activities of both Btk and Itk (37, 38) probably through the generation of inositol phosphates, which can bind the PH domains of Btk and Itk, targeting them to the cell membrane (39–41). Btk and Itk are then phosphorylated by Src superfamily kinases, resulting in their activation (37, 38, 42–46). Since Txk lacks a PH domain, either another mechanism mediates its cell membrane localization or this step is not essential for its activation.

Btk action is thought to be mediated, at least in part, by the downstream activation of PLC-γ2, the major PLC-γ isoform expressed in B cells (25, 26). In cells mutant for Btk, PLC-γ2 phosphorylation was reduced, leading to a defect in B cell receptor-mediated phosphoinositol hydrolysis and calcium influx. In T cells, absence of Itk results in decreased phosphorylation of PLC-γ1 and decreased calcium flux in response to TCR stimulation (27). Txk appears to function similarly to Itk in T cells as evidenced by the further impairment in PLC-γ1-mediated signaling responses in mice made deficient for both *Itk* and *txk* (24), and by the increase in tyrosine phosphorylation of PLC-γ1 and in intracellular calcium responses observed here in *txk* transgenic thymocytes. Moreover, Itk and Txk function specifically within the calcium signaling pathway, as TCR proximal signaling events such as phosphorylation of TCR-ζ and ZAP-70 are unaffected in *Itk*^{-/-} (27), *Itk*^{-/-} × *txk*^{-/-} (24), and *txk* transgenic mice (Fig. 4). Activation of Vav and Erk-2 is also unaffected in *txk* transgenic mice; however, decreased MAP kinase activation was noted in *Itk*^{-/-} × *txk*^{-/-} mice (24). Although Txk and Itk appear to perform similar functions in terms of enhancing PLC-γ1 phosphorylation and the calcium response to TCR cross-linking, endogenous Txk cannot replace Itk, since significant defects in PLC-γ1 phosphorylation and calcium flux are readily observed in *Itk*^{-/-} mice (27). However, we have shown that overexpres-

sion of Txk can, at least partially, restore the defect in thymocyte selection (Fig. 7) and calcium mobilization (Fig. 8 a) in *Itk*^{-/-} mice. One explanation for these results could be that Txk and Itk are functionally redundant but that endogenous Itk is expressed at significantly higher levels than endogenous Txk, such that Txk overexpression is required to correct the *Itk*^{-/-} defect. Alternatively, Txk may function inefficiently as a substitute for Itk, perhaps due to its lack of a PH domain. A similar example of partially redundant Src family tyrosine kinases in T cells was observed when expression of a constitutively active Fyn transgene in *lck*^{-/-} mice resulted in partial rescue of thymocyte development (47). It is also important to note that our results do not exclude the possibility that Txk indirectly enhances PLC-γ1 phosphorylation through some as yet unknown intermediary.

We also addressed whether overexpression of Txk could correct the defect in positive selection in *Itk*^{-/-} mice. In the absence of Itk, positive selection of thymocytes bearing a defined transgenic TCR (H-Y or AND) is severely reduced (21). Overexpression of Txk in *Itk*^{-/-} mice partially reversed this defect, suggesting that Txk could substitute for Itk. An additional observation made by Liao and Littman (21) and borne out here is the presence of an increased number of transgenic TCR^{hi} DN thymocytes in H-Y and AND transgenic *Itk*^{-/-} mice. Although the identity of these cells is unknown, their number decreased with Txk overexpression, suggesting a return toward a normal phenotype and a redundancy in function between Txk and Itk. Therefore, these experiments reveal a similar role for Txk and Itk in the signaling pathway required for positive selection.

Txk overexpression also influenced the efficiency of positive selection in the wild-type (*Itk*^{+/+}) background. This effect was seen only in TCR transgenic mice and therefore did not represent a generalized inhibition of positive selection, but rather seems to be due to the alteration in the signaling response of the TCR. In contrast to the rescuing effect of Txk overexpression in *Itk*^{-/-} mice, in wild-type (*Itk*^{+/+}) mice Txk overexpression resulted in the generation of fewer transgenic TCR^{hi} SP thymocytes in both H-Y TCR transgenic and AND TCR transgenic mice. If Txk

overexpression enhances TCR signaling as suggested by our biochemical and functional analysis, it might have been predicted that the efficiency of positive selection would increase in TCR transgenic mice, resulting in the generation of more rather than fewer transgenic TCR^{hi} SP thymocytes. Other genetic alterations that enhance TCR signaling have been shown to increase the efficiency of positive selection in TCR transgenic mice as assessed by criteria similar to those employed here. For example, in the absence of CD5, which exerts an inhibitory effect on TCR signaling, thymocytes are hyperresponsive to TCR cross-linking, and the efficiency of positive selection is enhanced in H-Y TCR transgenic mice (48). Similar effects on positive selection were observed in CD45^{+/-} mice as assessed in P14 TCR transgenic mice (49). However, negative selection was also enhanced in CD45^{+/-} mice as assessed by lymphocytic choriomeningitis virus infection (49). In our experiments, we observed fewer transgenic TCR^{hi} SP thymocytes in *txk* transgenic mice. This result could be explained by the failure of *txk* transgenic thymocytes to signal appropriately for positive selection, perhaps due to the delivery of an unbalanced TCR signal resulting from selective augmentation of the PLC- γ /calcium pathway relative to other downstream pathways such as the Ras pathway. Alternatively, the reduction in transgenic TCR^{hi} SP thymocytes in *txk* transgenic mice could be attributed to induction of cell death (negative selection) as a result of increased TCR signaling. In fact, negative selection has been shown to be particularly sensitive to alterations in calcium flux (50, 51). Elevated calcium responses have been correlated with negative selection in H-Y transgenic mice (50) and in TCR transgenic fetal thymic organ cultures treated with altered peptide ligands (52). In addition, in B cells, qualitatively dif-

ferent calcium signals can result in differential transcriptional responses (53, 54), suggesting that the kinetics of the calcium response can selectively influence the expression of specific genes.

Although negative selection was unaffected by Txk overexpression in H-Y transgenic male mice (data not shown), increased cell death may still account for the apparent decrease in positive selection seen in H-Y female and AND transgenic mice. Negative selection occurs early in H-Y male thymocytes, before the DP stage (55), and enhancement of negative selection has been difficult to demonstrate using this particular system. Since we observed normal thymocyte cellularity and increased numbers of transgenic TCR^{hi} DP thymocytes in *txk* transgenic \times TCR transgenic mice, enhanced cell death may be occurring at a later stage (e.g., the early SP stage [56]). Consistent with this idea, a higher percentage of thymocytes from *txk* transgenic mice undergoes apoptosis when cultured either in the absence or presence of stimulating antibodies (anti-TCR plus anti-CD28) compared with thymocytes from non-*txk* transgenic mice (data not shown). Although these findings are subject to other interpretations, we favor the hypothesis that increased apoptosis of *txk* transgenic thymocytes cultured in the absence of stimulating antibodies reflects the outcome of signals generated in vivo. Indeed, PLC- γ 1 was found to be hyperphosphorylated in ex vivo thymocytes from *txk* transgenic mice, indicating enhanced signaling in vivo.

In conclusion, our results identify a specific role for Txk in the TCR signaling pathway leading to calcium mobilization, and demonstrate that Txk can influence the efficiency of positive selection. In addition, these findings reveal a similarity in function for the Tec family kinases Txk and Itk in T cells.

We gratefully acknowledge the generosity of Lawrence Samelson (anti-Lck and anti-ZAP-70) and Daniel Littman (*itk*^{-/-} mice). Elizabeth Shores, Lawrence Samelson, Ronald Germain, B.J. Fowlkes, Weiguang Zhang, David Weist, and Kenneth Katz provided helpful advice. We would also like to thank Sharon Campbell, Kin Lui, Dalal El Khoury, Larry Duckett, and Ruth Swofford for technical assistance.

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Submitted: 22 April 1999 Revised: 20 August 1999 Accepted: 13 September 1999

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