# T Cell Receptor–initiated Calcium Release Is Uncoupled from Capacitative Calcium Entry in Itk-deficient T Cells

By Karen-Qianye Liu,[§] Stephen C. Bunnell,[‡] Christine B. Gurniak,[*] and Leslie J. Berg[*]

*From the [*]Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655; the [‡]Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138; and the [§]Program of Immunology, Division of Medical Sciences, Harvard University, Boston, Massachusetts 02115*

**Summary**

Itk, a Tec family tyrosine kinase, plays an important but as yet undefined role in T cell receptor (TCR) signaling. Here we show that T cells from Itk-deficient mice have a TCR-proximal signaling defect, resulting in defective interleukin 2 secretion. Upon TCR stimulation, $Itk^{-/-}$ T cells release normal amounts of calcium from intracellular stores, but fail to open plasma membrane calcium channels. Since thapsigargin-induced store depletion triggers normal calcium entry in $Itk^{-/-}$ T cells, an impaired biochemical link between store depletion and channel opening is unlikely to be responsible for this defect. Biochemical studies indicate that TCR-induced inositol 1,4,5 tris-phosphate (IP3) generation and phospholipase C γ1 tyrosine phosphorylation are substantially reduced in $Itk^{-/-}$ T cells. In contrast, TCR-ζ and ZAP-70 are phosphorylated normally, suggesting that Itk functions downstream of, or in parallel to, ZAP-70 to facilitate TCR-induced IP3 production. These findings support a model in which quantitative differences in cytosolic IP3 trigger distinct responses, and in which only high concentrations of IP3 trigger the influx of extracellular calcium.

Key words: T cell activation • knockout mouse • Tec family tyrosine kinase • calcium flux • T cell receptor signaling

T cell activation is a complex process involving a number of independent signaling pathways. Of these, the best understood is the signaling downstream of the TCR (for review see reference 1). Biochemical as well as genetic studies have identified many of the important signaling molecules activated by TCR stimulation. For instance, the tyrosine kinases ZAP-70 and Lck are known to play a critical role during T cell activation. Evidence also supports a role for the Ras/Map kinase cascade, as well as the phospholipid modifying enzyme, phosphatidyl inositol–3 kinase (PI3K). To date, the precise role of each of these signaling branches, as well as the mechanism(s) that integrate these diverse signals, has not been completely elucidated. However, it has long been known that pharmacological agents such as phorbol esters plus calcium ionophores can bypass T cell surface receptors and directly activate resting T cells (2). This phenomenon clearly pinpoints the critical role of protein kinase C and calcium signaling in T cell activation.

The Tec family tyrosine kinases have been implicated in antigen receptor signaling in a variety of hematopoietic cell types. Itk, expressed in T cells and natural killer cells (3, 4), is tyrosine phosphorylated in response to cross-linking of the TCR (5, 6), CD28 (7), or CD2 (8). A closely related family member, Btk, is expressed in B cells and mast cells; immunodeficiency diseases in humans (XLA; references 9–11) and mice (*xid*; references 12, 13) result from mutations in the Btk gene, and are characterized by defects in B cell receptor (BCR) signaling (14–17). Furthermore, in Btk-deficient chicken DT40 B cells, the antigen receptor–induced tyrosine phosphorylation of the phospholipase C (PLC)[1] isoform PLC-γ2 is substantially reduced, resulting in the loss both of BCR-coupled phosphatidylinositol hydrolysis and of calcium mobilization (18). Our studies suggested that Itk might play an analogous role in TCR signaling in T cells.

To test this possibility, we generated a line of Itk-deficient mice. Previous studies have shown that Itk is important for normal T cell development in the thymus and for

[1]*Abbreviations used in this paper:* ES, embryonic stem; PI3, inositol 1,4,5 tris-phosphate; PLC, phospholipase C.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/05/1721/07 $2.00
Volume 187, Number 10, May 18, 1998 1721–1727
http://www.jem.org

normal T cell activation (19). However, the precise role of Itk in TCR signal transduction has not yet been described. Our studies have extended these previous observations by demonstrating biochemical defects in $Itk^{-/-}$ T cells. We show that IL-2 secretion is dramatically reduced after TCR stimulation of $Itk^{-/-}$ T cells, and that this defect results, at least in part, from impaired calcium signaling. We also show that the failure to generate a sustained calcium flux in $Itk^{-/-}$ T cells is correlated with a dramatic reduction in TCR-induced inositol 1,4,5 tris-phosphate (IP3) generation and with reduced tyrosine phosphorylation of PLC-γ1. In contrast, TCR-induced tyrosine phosphorylation of ZAP-70 and the TCR-ζ chain are unaffected. These data indicate that Itk is downstream of or parallel to ZAP-70, and, furthermore, that Itk is an important component of the signaling pathway leading to the sustained elevation of cytoplasmic free calcium necessary for the activation of transcription factors such as c-rel and the nuclear factor of activated T cells (NFAT).

## Materials and Methods

*Generation of Itk-deficient Mice.* A murine strain 129 genomic clone encoding the Itk kinase domain (exons 9, 10, 11, 12, and 13 [see Fig. 1]) was isolated. A 2.6-kb HindIII fragment extending from the middle of exon 10 to beyond exon 12 was replaced by the 1.8-kb pGK-neo cassette; these deleted sequences encode a portion of the Itk kinase domain (amino acids 476–543). The targeting vector also contained 4.8 kb and 4.2 kb of flanking sequences at the 5′ and 3′ ends, respectively, and a 2.0-kb thymidine kinase cassette (pGK-tk). The construct was linearized and transfected into J1 (gift of Dr. A. Sharpe, Harvard Medical School, Boston, MA) and CCE (gift of Dr. E. Robertson, Harvard University, Cambridge, MA) embryonic stem (ES) cell lines. 360 clones resistant to Geneticin (Sigma Chemical Co., St. Louis, MO) and FIAU [1-(2′-deoxy-2′-fluro-β-d arabinofuranosyl)-5-iodouracil; gift of Dr. A. McMahon, Harvard University, Cambridge, MA] were screened, yielding two homologous integrants, one from each ES cell line. The ES clones were injected into C57Bl/6 blastocysts to generate chimeric mice. Chimeras (male) were backcrossed to C57Bl/6 females and progeny were screened for the mutated Itk allele. Heterozygous $Itk^{+/-}$ mice were intercrossed to generate $Itk^{-/-}$ mice. Identical phenotypes were observed for $Itk^{-/-}$ mice generated from both of the two original ES cell lines; these lines were used interchangeably. Mice were maintained under specific pathogen-free conditions.

*Cell Purification.* For $CD4^+$ T cell purification, lymph node (inguinal, axillary, brachial, mandibular, and mesenteric) cells from multiple wild-type or Itk-deficient mice were isolated and were purified on columns (Biotex Laboratories, Alberta, Canada) according to the manufacturer's protocol. The resulting cells were 90–97% pure ($Itk^{+/+}$ or $Itk^{+/-}$) or 80–90% pure ($Itk^{-/-}$) $CD4^+$ T cells.

*Antibodies and Flow Cytometry Analysis.* Before antibody staining $10^6$ lymph node cells were plated in wells precoated with 5 μg/ml goat anti–hamster IgG(H+L) followed by 5 μg/ml hamster anti-mouse CD3ε (PharMingen, San Diego, CA). After 18–20 h of stimulation, cells were stained with the following antibodies: biotinylated anti-CD3ε (PharMingen, San Diego, CA), biotinylated anti-CD69 (PharMingen), biotinylated anti-CD25 (PharMingen), biotinylated anti-CD62L (PharMingen), FITC-conjugated anti–TCR-α/β (PharMingen), PE-conjugated anti-CD4 (GIBCO BRL, Bethesda, MD), and CD8-Red (GIBCO BRL); secondary reagent was streptavidin-FITC (Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed on a FACScan® using the CellQuest software (Becton Dickinson, Mountain View, CA). Immunoprecipitations and Western blotting were done with antibodies to the following proteins: Itk (monoclonal antibodies 7F10 and 10B2; reference 6), Tec (gift of Dr. B. Tang, St. Jude Children's Research Hospital, Memphis, TN), TCR-ζ (Santa Cruz Biotech, Santa Cruz, CA), ZAP-70 (Santa Cruz Biotech), and PLC-γ1 (Santa Cruz Biotech and Upstate Biotechnology, Lake Placid, NY). Anti-phosphotyrosine antibody 4G10 was a gift from Dr. B. Druker (Oregon Health Sciences Center, Portland, OR).

*TCR Stimulation and IL-2 Assays.* For T cell stimulations, $5 \times 10^4$ purified $CD4^+$ T cells were plated in wells precoated with goat anti–hamster antibody (5 μg/ml) followed by hamster anti–mouse CD3ε (0.5 μg/ml). Where indicated, a 1:8 dilution (determined to be saturating by cell surface staining) of anti-CD28 antibody supernatant (37N.D1; a gift from Dr. J. Allison (University of California at Berkeley, Berkeley, CA; reference 20) was added. As a control, cells were also stimulated with PMA (1 ng/ml) plus ionomycin (500 ng/ml). After 24 h of stimulation, the supernatants were assessed for IL-2 concentration using HT-2 indicator cells as previously described (21).

*Calcium Flux.* $5 \times 10^6$/ml purified $CD4^+$ cells were incubated with 3 μg/ml fluo-3, 5 μg/ml Fura-Red, 0.5% pluronic acid (Molecular Probes, Eugene, OR), and 2% FCS in RPMI for 30–50 min. Dye-loaded cells were washed twice with RPMI and left at room temperature for 30 min in the dark before use. $1.5 \times 10^6$ (in 300 μl) dye-loaded cells were placed in 1 ml of serum-free RPMI and warmed to 37°C. Stimulated cells were analyzed on a FACScan®. The mean fluorescence ratio (FL-1:FL-3) was calculated using the FACSAssistant® program.

*Immunoprecipitation and Western Blotting.* Purified $CD4^+$ T cells were incubated in serum-free RPMI for 2 h at 37°C before stimulation. Cells were then washed twice, resuspended in 120 μl of RPMI containing 25 μg/ml biotinylated anti-CD3ε antibody, and incubated on ice for 10 min. 50 μg/ml of streptavidin (Sigma Chemical Co.) was then added, and cells were incubated at 37°C for the indicated times; signaling was terminated by diluting the samples into 1 ml of ice-cold PBS containing 20 mM NaF and 1 mM $Na_3VO_4$. Cells were then lysed in lysis buffer (0.5% Brij97, 20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 20 mM NaF, and 1 mM $Na_3VO_4$) supplemented with protease inhibitors. Immunoprecipitations were done with lysate from $3 \times 10^6$ cells. Immunoprecipitated proteins were resolved by 8 or 12% SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and blocked and probed as previously described (22).

*Analysis of IP3 Release.* $8 \times 10^6$ purified $CD4^+$ T cells were starved in serum-free RPMI for 1 h at 37°C before stimulation. Cells were stimulated as for immunoprecipitation studies, but at $3.2 \times 10^7$/ml; signaling was terminated by adding 50 μl ice-cold 100% Trichloroacetic acid. IP3 levels were assessed using the IP3 Radioreceptor Assay Kit (NEN Life Science Products, Boston, MA) following the manufacturer's protocol. Controls included medium alone or mock-stimulated (no primary antibody) cells.

## Results and Discussion

To determine the function of Itk in T cell signaling, we generated Itk-deficient mice by gene targeting. Exons of the Itk gene encoding critical regions of the kinase domain

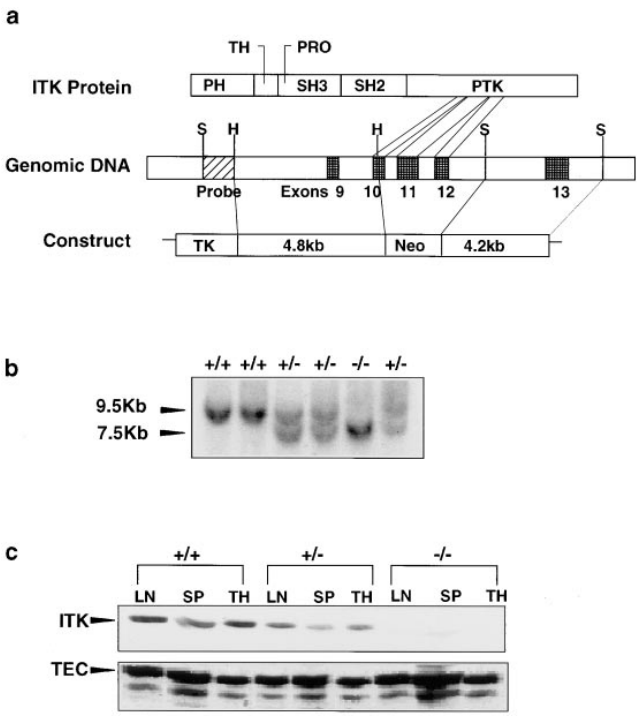


**Figure 1.** Disruption of the Itk gene. (*a*) Domain structure of the Itk protein and physical map of the Itk genomic clone. Relative positions of exons 9–13 are depicted in checkered boxes. *S*, Sac I; *H*, HindIII; *TH*, Tec homology domain; *PH*, pleckstrin homology domain; *PRO*, proline-rich region; *TK*, thymidine kinase cassette; *Neo*, neomycin$^R$ cassette. The probe used for Southern blot analysis is depicted by a hatched box. (*b*) Southern blot of tail DNA from Itk$^{+/+}$ (+/+), Itk$^{+/-}$ (+/−), and Itk$^{-/-}$ (−/−) mice. DNA was digested with SacI, which yields a 9.5-kb band from the wild-type allele and a 7.5-kb band from the mutant allele. (*c*) Total lysate of $5 \times 10^6$ cells from lymph node (*LN*), spleen (*SP*), or thymus (*TH*) from the indicated mice were immunoblotted with anti-Itk monoclonal antibody (*top panel*); filter was stripped and reprobed with anti-Tec antibody (*bottom panel*).

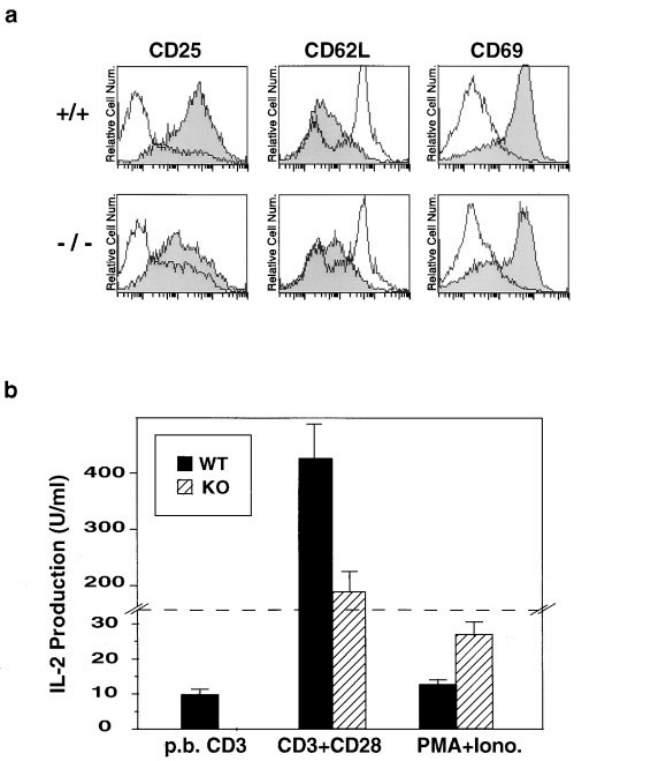


**Figure 2.** Defective IL-2 production by Itk$^{-/-}$ T cells. (*a*) Total lymph node cells were analyzed for expression of CD25, CD69, and CD62L after 18–20 h of activation by plate-bound anti-CD3ε antibody. Histogram of live gated CD4$^+$ cells are shown. Open histograms show expression of markers when cultured in media alone; filled histograms show expression of markers after stimulation. (*b*) Purified CD4$^+$ T cells were stimulated with plate-bound anti-CD3ε antibody (*p.b. CD3*), anti-CD3ε plus anti-CD28 antibodies (*CD3+CD28*), or PMA plus ionomycin (*PMA+Iono*), and analyzed for IL-2 secretion after 24 h. Graph is representative of four similar experiments.

were deleted, and replaced with the neo$^R$ cassette (Fig. 1 *a*). Mice homozygous for the mutated Itk allele (Itk$^{-/-}$; see Fig. 1 *b*) fail to express any Itk protein in lymph nodes, spleen, or thymus (Fig. 1 *c*). Consistent with a previous report on Itk-deficient mice (19), T cell development is abnormal in the absence of the Itk protein. Specifically, the size and general histology of the thymus are normal, but CD4$^+$ T cell maturation is diminished in Itk$^{-/-}$ mice. The number of CD4$^+$ T cells in peripheral lymphoid organs is reduced to ~50% of that found in wild-type mice; in contrast, the number of CD8$^+$ T cells is unchanged (data not shown).

To investigate the role of Itk in TCR signaling, we examined the responses of Itk$^{-/-}$ CD4$^+$ T cells to TCR stimulation. First, Itk$^{-/-}$ lymph node cells were stimulated with plate-bound anti-CD3 antibody, and cells were assessed 18–20 h later for changes in surface marker expression. CD25 and CD69 were upregulated and CD62L (L-selectin) was downregulated after TCR stimulation on both mutant and wild-type cells (Fig. 2 *a*). The only difference we consistently observed was a somewhat higher degree of CD25 upregulation on Itk$^{+/+}$ cells compared with Itk$^{-/-}$ cells; this difference is most likely due to the IL-2–induced increase in IL-2 receptor α chain (CD25) expression, which is lacking in Itk$^{-/-}$ T cells (see below). These data indicate that the TCR-dependent signaling pathways leading to changes in activation marker and adhesion molecule expression are intact in Itk$^{-/-}$ T cells. Second, we examined IL-2 secretion in response to TCR stimulation. When purified CD4$^+$ Itk$^{-/-}$ T cells were stimulated with anti-CD3 antibody, no IL-2 could be detected in the supernatants after overnight culture (Fig. 2 *b*). Stimulation with anti-CD3 plus anti-CD28 antibodies, which dramatically enhances IL-2 production from wild-type cells, resulted in detectable, but still reduced, IL-2 secretion from Itk$^{-/-}$ T cells. This signaling defect is proximal to the TCR, as stimulation with PMA plus ionomycin, which bypasses the early stages of TCR signaling, elicits comparable IL-2 secretion by Itk$^{-/-}$ and wild-type T cells.

The functional defect in IL-2 production after stimulation of Itk$^{-/-}$ T cells suggested a defect in the TCR signaling pathway leading to IL-2 transcription. Since IL-2 promoter activity is highly dependent on TCR-induced

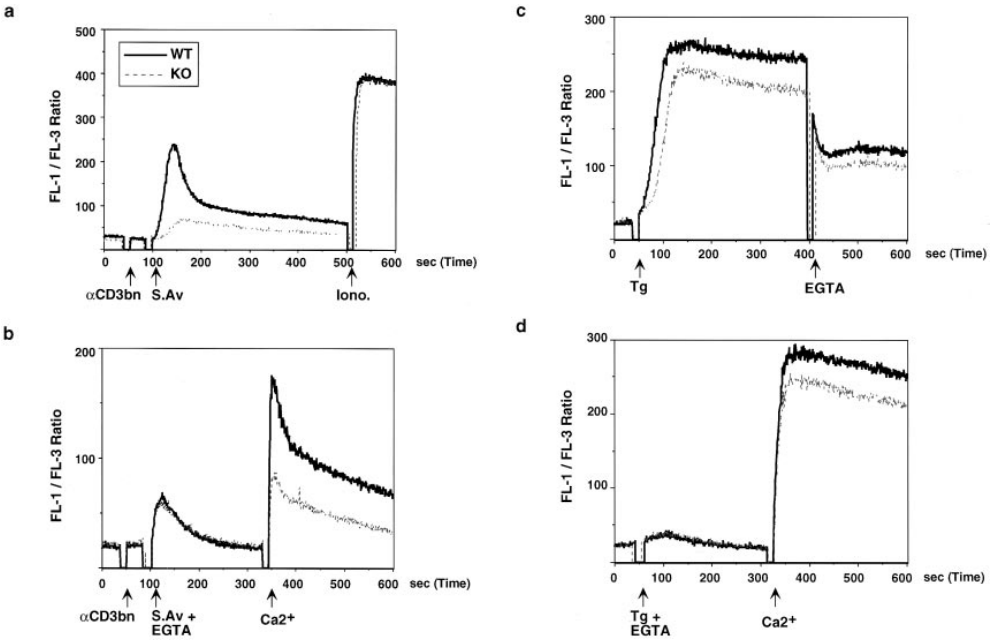


**Figure 3.** Defective calcium flux response after TCR stimulation of Itk$^{-/-}$ T cells. Purified CD4$^{+}$ T cells were stimulated and monitored for 600 s after gating on live cells. The intracellular calcium concentration is plotted as the FL1/FL3 ratio over time. (*a*) Calcium response after CD3 cross-linking. 500 ng/ml ionomycin was added as a control at 500 s to assess the efficiency of dye loading. (*b*) Calcium response after CD3 cross-linking in the presence of extracellular EGTA (0.5 mM) sufficient to chelate the calcium present in the RPMI media (~0.4 mM). Extracellular calcium was added back to a final concentration of 0.5 mM free $Ca^{2+}$ midway through the experiment. (*c*) Intracellular calcium elevation in response to 1 μM thapsigargin (*Tg*). Extracellular EGTA was added midway through the experiment. (*d*) Thapsigargin (*Tg*)-induced calcium elevation in the presence of EGTA (0.5 mM). *Solid lines*, control (Itk$^{+/+}$ and Itk$^{+/-}$) cells; *dashed lines*, Itk$^{-/-}$ cells.

sustained increases in cytoplasmic free calcium (23–25), we examined calcium responses after TCR stimulation of CD4$^{+}$ T cells. In wild-type cells, TCR cross-linking elicits the expected large, transient peak of increased calcium in the cytoplasm, followed by a sustained elevation in intracellular calcium (Fig. 3 *a*). In contrast, Itk$^{-/-}$ T cells have a substantially reduced initial peak of elevated cytoplasmic calcium and do not sustain elevated calcium levels. This response requires high concentrations of the stimulating anti-CD3ε antibody (10 μg/ml); when lower concentrations of antibody are used, wild-type T cells display a slower and more flattened elevation in intracellular calcium, whereas Itk$^{-/-}$ T cells fail to respond altogether (data not shown). These differences are not due to defective dye loading in the mutant cells, as ionomycin induces equivalent large increases in cytoplasmic calcium in both cell types (Fig. 3 *a*). Although the defective calcium influx observed with high concentrations of stimulating antibody is novel, the more severe defect observed with lower concentrations of stimulating antibody parallels the defect observed upon surface immunoglobulin cross-linking of Btk$^{-/-}$ chicken B cells (18). It is not clear if the partial defect observed at high concentrations of stimulating antibody would also be observed in the B cell system.

In normal cells, the overall pattern of calcium elevation results from two components of the calcium response. The initial transient peak is due to the release of calcium from intracellular stores (26); the resulting depletion of these stores triggers the opening of the plasma membrane calcium channels, generating a sustained elevation in intracellular calcium (27). To determine whether Itk$^{-/-}$ T cells are defective in one or both of these components of the calcium response, we chelated the extracellular calcium with EGTA, eliminating the influx of calcium and thereby revealing the release of calcium from intracellular stores. In the presence of EGTA, the transient calcium increase induced by TCR cross-linking is similar in both wild-type and mutant cells (Fig. 3 *b*), suggesting that the intracellular calcium stores are released (and therefore depleted) to a similar extent in both cell types. Nonetheless, when extracellular $Ca^{2+}$ is restored, allowing the influx of calcium across the plasma membrane, wild-type cells sustain a substantially higher level of intracellular calcium than do mutant cells. These data indicate that the signaling pathways opening plasma membrane calcium channels in response to store depletion are defective in Itk$^{-/-}$ T cells, resulting in the reduced level of calcium elevation after TCR stimulation. Since sustained calcium elevation is required for the sustained nuclear translocation of nuclear factor of activated T cells (NFAT) and for IL-2 transcription (23–25), the failure of Itk$^{-/-}$ cells to sustain intracellular calcium levels is likely to be a direct cause of the reduced IL-2 production observed after TCR stimulation.

To determine whether the depletion of intracellular calcium stores is competent to induce the opening of plasma membrane calcium channels, wild-type and mutant T cells were treated with the drug thapsigargin. Thapsigargin, a specific inhibitor of the $Ca^{2+}$-ATPase in the endoplasmic reticulum membrane, prevents the uptake of cytosolic calcium and results in a leak that depletes intracellular calcium

stores and triggers calcium entry (28, 29). In contrast to anti-CD3 antibody cross-linking, thapsigargin treatment initiates a substantial calcium influx in both wild-type and mutant T cells, demonstrating that store-operated calcium entry is normal in Itk$^{-/-}$ T cells (Fig. 3 *c*). It is possible that distinct depletion-sensitive signaling pathways are induced depending on the mechanism of store depletion; however, we favor a model in which the channel-operating calcium stores are depleted to a lesser extent in the Itk$^{-/-}$ cells. This latter hypothesis is consistent with the reduced production of IP3 observed after TCR stimulation of Itk$^{-/-}$ T cells (see below).

In the presence of extracellular EGTA, thapsigargin induces similar levels of calcium elevation in mutant and wild-type T cell (Fig. 3 *d*), suggesting that the sizes of the thapsigargin-sensitive intracellular calcium stores are similar in both cell types. Notably, the thapsigargin-stimulated calcium release observed in these naive T cells is unexpectedly small, yet capable of stimulating calcium influx. In light of this result, it is surprising that the large calcium release observed upon TCR stimulation in Itk-deficient T cells fails to trigger calcium influx. This apparent contradiction could be explained in at least three ways. First, the extent of calcium elevation in the presence of thapsigargin may be a poor measure of store depletion, as rates of store refilling may differ between wild-type and Itk$^{-/-}$ T cells. Second the similar extent of store release observed between wild-type and Itk$^{-/-}$ cells may underestimate the actual difference in total calcium released if the rate at which calcium is cleared from the cyotosol increases as the concentration of cytosolic calcium increases; this phenomenon has been documented previously in T cells (30). Third, it is possible that the thapsigargin-sensitive intracellular stores are a distinct and small subset of the TCR-coupled intracellular calcium stores, and are uniquely competent to trigger calcium influx in response to depletion. In this latter case, Itk would be required to couple the TCR to these specific channel-operating calcium stores.

It should be noted that the amplitude of the thapsigargin-induced calcium elevation is always slightly diminished in Itk-deficient T cells (Fig. 3 *c*). When purified cell populations are gated to exclude contaminating CD4$^-$ cells, the same difference is observed (data not shown); therefore, this difference is not due to disparities in the purities of the CD4$^+$ T cell preparations from mutant and wild-type lymph nodes. The absence of Itk could affect peak intracellular calcium levels by multiple mechanisms, potentially reducing the frequency of calcium channel opening, decreasing the electrochemical gradient driving calcium entry, or by facilitating the upregulation of the plasma membrane calcium pump. At present we do not know which, if any, of these mechanisms result in the reduction in maximum intracellular calcium levels induced by thapsigargin.

TCR-induced calcium responses in T cells are initiated by the second messenger, IP3. IP3 is generated by the PLC-mediated hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2). After PIP2 cleavage, IP3 diffuses through the cytoplasm and binds to IP3 receptors, inducing the release of calcium from internal stores (for review see reference 31). In addition, IP3 is thought to play a role in the subsequent calcium signal that causes the opening of the plasma membrane calcium channels (32). Therefore, we assessed the levels of IP3 generated after TCR stimulation of wild-type and Itk$^{-/-}$ T cells (Fig. 4 *a*). In wild-type cells, IP3 levels begin to rise by 45 s after TCR stimulation, remain at peak levels between 1 and 2 min, and return to basal levels by 5 min (Fig. 4 *a* and data not shown). In contrast, no significant increase in IP3 levels over background can be detected in Itk$^{-/-}$ T cells at any time point (Fig. 4 and data not shown). Parekh et al. have demonstrated that calcium release can be triggered by IP3 concentrations 50-fold below those required to trigger calcium influx (32). The minimal calcium-releasing doses of IP3 identified by Parekh are at the limit of detection of our assay system, and it is likely that TCR stimulation in Itk$^{-/-}$ T cells actually induces small amounts of IP3 that activate calcium release without triggering calcium influx. It is unclear why this threshold effect exists. As proposed by

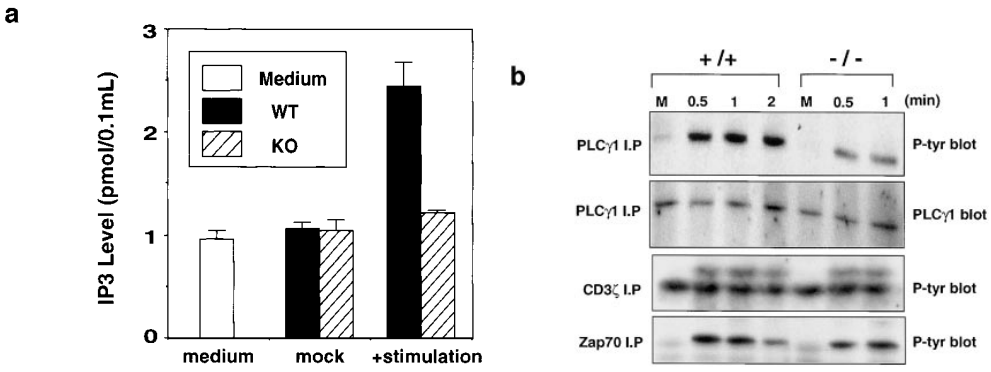


**Figure 4.** Reduced PLC-γ1 phosphorylation and IP3 production by activated Itk$^{-/-}$ T cells. (*a*) Purified CD4$^+$ T cells from wild-type (*WT*) and Itk$^{-/-}$ (*KO*) mice were incubated in the presence (+*stimulation*) or absence (*mock*) of anti-CD3ε antibody for 1.5 min; cell lysates were prepared and analyzed for IP3 levels. A value obtained from medium in the absence of cells is shown (*medium*). Averages and standard deviations were calculated from two to three independent experiments. (*b*) Purified CD4$^+$ T cells from wild-type (+/+) and Itk$^{-/-}$ (−/−) mice were stimulated by anti-CD3ε antibody cross-linking. PLC-γ1, CD3ζ, and ZAP-70 were immunoprecipitated from the cell lysates and immunoblotted for phosphotyrosine ($n$ = 3). In the case of PLC-γ1, the membrane was reprobed to detect the immunoprecipitated protein.

Parekh et al., it could result either from a low affinity IP3-receptor present on a specialized channel-coupled calcium store, or from the need for IP3 concentrations sufficient to allow diffusion to outpace the catabolism of IP3 (32).

The PLC isoform PLC-γ1 is abundant in T cells and tyrosine phosphorylated after TCR stimulation. Since this PLC isoform is activated by tyrosine phosphorylation (33), we examined whether reduced tyrosine phosphorylation of PLC-γ1 could account for the defective TCR-induced IP3 production in Itk$^{-/-}$ T cells. As shown in Fig. 4 *b*, immunoblotting of PLC-γ1 with an antiphosphotyrosine antibody indicated that the tyrosine phosphorylation of PLC-γ1 from stimulated Itk$^{-/-}$ T cells is markedly reduced at 0.5 and 1 min. This reduced phosphorylation is not due to decreased levels of PLC-γ1, as demonstrated by anti-PLC-γ1 immunoblotting (Fig. 4 *b*). In contrast, the TCR-induced phosphorylation of the receptor-proximal signaling molecules ZAP-70 and TCR-ζ is similar in both wild-type and Itk$^{-/-}$ T cells. Furthermore, the overall pattern of tyrosine phosphorylation induced by TCR stimulation, with minor exceptions, is not markedly different between Itk$^{+/+}$ and Itk$^{-/-}$ T cells (data not shown); in addition, some downstream functions of TCR signaling are intact (e.g., changes in activation marker expression, see above). Since ZAP-70 activation is critical for TCR signaling (34, 35), our data suggest that ZAP-70 activation is normal in the absence of Itk. These data indicate that Itk is not upstream of either TCR-ζ or ZAP-70 phosphorylation, but is required for optimal PLC-γ1 activation after TCR cross-linking. This placement of Itk relative to ZAP-70 is consistent with that proposed by Kurosaki et al. for Btk and Syk (17).

Here we demonstrate that Itk$^{-/-}$ T cells have a defect in TCR signaling that leads to reduced IL-2 secretion after TCR cross-linking. Although we have not ruled out additional signaling defects in Itk$^{-/-}$ T cells, the impaired influx of calcium across the plasma membrane is likely to be responsible for the loss of TCR-induced IL-2 production. Since calcium influx enables the sustained increases in cytoplasmic calcium required for transcription factor activation (23–25), this defect could explain the inability of Itk$^{-/-}$ T cells to secrete IL-2. As calcium release from intracellular stores appears normal in the Itk$^{-/-}$ T cells, these data suggest that Itk might be involved in the signaling pathways coupling store depletion to calcium influx. However, we found this explanation unlikely because thapsigargin induces calcium influx in the absence of Itk. This finding suggests that a functional depletion-sensing pathway can be decoupled from calcium release. Defective coupling could result from impaired PLC-γ1 activation in Itk$^{-/-}$ T cells by at least two mechanisms. The dramatically reduced production of IP3 after TCR stimulation in Itk$^{-/-}$ T cells could fail to trigger a low affinity IP3 receptor on a channel-operating subset of intracellular calcium stores, while fully activating calcium release from the remaining stores. Alternatively, the low levels of IP3 produced in the Itk$^{-/-}$ T cells could fail to deplete intracellular calcium stores to the extent required to trigger calcium influx. In this case, we would be forced to conclude that the observed store release is a poor measure of actual store depletion, either because we are underestimating release in the wild-type T cells, or because the rates at which the stores refill differ between the wild-type and Itk$^{-/-}$ T cells. We believe that this is the first demonstration that the TCR-induced influx of extracellular calcium can be uncoupled from the TCR-induced release of intracellular calcium, while preserving functional, depletion-sensitive calcium influx mechanisms. Thus, these experiments suggest that Itk$^{-/-}$ T cells will provide an interesting model system for detailed studies of the precise mechanisms regulating intracellular calcium levels.

We thank Dr. Arlene Sharpe and Dr. Elizabeth Robertson for embryonic stem cell lines and technical advice; Jennifer Noble for generating the chimeric mice; Dr. Brian Druker for providing 4G10 antibody; Dr. B. Tang for providing the anti-Tec antibody; Dr. Andrew McMahon for providing FIAU; and Dr. Daniel Thomis for helpful discussions and critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (AI-37584) and the Council for Tobacco Research-USA, Inc.

Address correspondence to Leslie J. Berg, Department of Pathology, University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, MA 01655. Phone: 508-856-8371; Fax: 508-856-8372; E-mail: leslie.berg@ummed.edu

Christine Gurniak's current address is Mouse Biology Programme/Monterotondo EMBL, European Molecular Biology Laboratory, Meyerhofstrasse, Postfach 10.2209, 69012 Heidelberg, Germany.

*Received for publication 3 March 1998.*

## References

1. Wange, R.L., and L.E. Samelson. 1996. Complex complexes: signaling at the TCR. *Immunity*. 5:197–205.
2. Weiss, A., R.L. Wiskocil, and J.D. Stobo. 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. *J. Immunol.* 133:123–128.

3. Heyeck, S.D., and L.J. Berg. 1993. Developmental regulation of a murine T cell–specific tyrosine kinase gene, Tsk. *Proc. Natl. Acad. Sci. USA*. 90:669–673.
4. Teng, J., X. Liu, G. Mills, and B. Dupont. 1996. CD28-mediated cytotoxicity by the human leukemic NK cell line YT involves tyrosine phosphorylation, activation of phosphatidylinositol 3-kinase, and protein kinase C. *J. Immunol*. 156:3222–3232.
5. Gibson, S., A. August, Y. Kawakami, T. Kawakami, B. Dupont, and G.B. Mills. 1996. The EMT/ITK/TSK (EMT) tyrosine kinase is activated during TCR signaling. *J. Immunol*. 156:2716–2722.
6. Heyeck, S.D., H.M. Wilcox, S.C. Bunnell, and L.J. Berg. 1997. Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity. *J. Biol. Chem*. 272:25401–25408.
7. Gibson, S., A. August, D. Branch, B. Dupont, and G.B. Mills. 1996. Functional Lck is required for optimal CD28-mediated activation of the Tec family tyrosine kinase Emt/Itk. *J. Biol. Chem*. 271:7079–7083.
8. King, P.D., A. Sadra, A. Han, X.-R. Liu, R. Sunder-Plassmann, E.L. Reinherz, and B. Dupont. 1996. CD2 signaling in T cells involves tyrosine phosphorylation and activation of the Tec family kinase, Emt/Itk/Tsk. *Int. Immunol*. 8:1707–1714.
9. Conley, M., O. Parolini, J. Rohrer, and D. Campana. 1994. X-linked agammaglobulinemia: new approaches to old questions based on the identification of the defective gene. *Immunol. Rev*. 138:5–21.
10. Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarstrom, C. Kinnon, R. Levinsky, M. Bobrow, et al. 1993. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein tyrosine kinases. *Nature*. 361:226–232.
11. Tsukada, S., D.C. Saffran, D.J. Rawlings, O. Parolini, R.C. Allen, I. Klisak, R.S. Sparkes, H. Kubagawa, T. Mohandas, S. Quan, et al. 1993. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 72:279–290.
12. Rawlings, D.J., D.C. Saffran, S. Tsukada, D.A. Largaespada, J.C. Grimaldi, L. Cohen, R.N. Mohr, J.F. Bazan, M. Howard, N.G. Copeland, et al. 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science*. 261:358–361.
13. Thomas, J.D., P. Sideras, C.I.E. Smith, I. Vorechovsky, V. Chapman, and W.E. Paul. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science*. 261:355–358.
14. Desiderio, S. 1997. Role of Btk in B cell development and signaling. *Curr. Opin. Immunol*. 9:534–540.
15. Aoki, Y., K.J. Isselbacher, and S. Pillai. 1994. Bruton's tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells. *Proc. Natl. Acad. Sci. USA*. 91:10606–10609.
16. Rawlings, D.J., A.M. Scharenberg, H. Park, M.I. Wahl, S. Lin, R.M. Kato, A.-C. Fluckiger, O.N. Witte, and J.-P. Kinet. 1996. Activation of BTK by a phosphorylation mechanism initiated by Src family kinases. *Science*. 271:822–825.
17. Kurosaki, T., and M. Kurosaki. 1997. Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. *J. Biol. Chem*. 272:15595–15598.
18. Takata, M., and T. Kurosaki. 1996. A role for Bruton's tyrosine kinase in B cell antigen receptor–mediated activation of phospholipase C-γ2. *J. Exp. Med*. 184:31–40.
19. Liao, X.C., and D.R. Littman. 1995. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. *Immunity*. 3:757–769.
20. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T cell clones. *Nature*. 356:607–609.
21. Yelon, D., L.M. Spain, K. Lim, and L.J. Berg. 1996. Alterations in CD4 dependence accompany T cell development and differentiation. *Int. Immunol*. 8:1077–1090.
22. Bunnell, S.C., P.A. Henry, R. Kolluri, T. Kirchhausen, R. Rickles, and L.J. Berg. 1996. Identification of Itk/Tsk SH3 domain ligands. *J. Biol. Chem*. 271:25646–25656.
23. Wacholtz, M., and P. Lipsky. 1993. Anti-CD3-stimulated $Ca^{2+}$ signal in individual human peripheral T cells. Activation correlates with a sustained increase in intracellular $Ca^{2+}$. *J. Immunol*. 150:5338–5349.
24. Timmerman, L., N. Clipstone, S. Ho, J. Northrop, and G. Crabtree. 1996. Rapid shuttling of NF-AT in discrimination of $Ca^{2+}$ signals and immunosuppression. *Nature*. 383:837–840.
25. Dolmetsch, R., R. Lewis, C. Goodnow, and J. Healy. 1997. Differential activation of transcription factors induced by $Ca^{2+}$ response amplitude and duration. *Nature*. 386:855–858.
26. Berridge, M. 1993. Inositol trisphosphate and calcium signaling. *Nature*. 361:315–325.
27. Putney, J.W., Jr., and G.S. Bird. 1993. The signal for capacitative calcium entry. *Cell*. 75:199–201.
28. Gouy, H., D. Cefai, S.B. Christensen, P. Debre, and G. Bismuth. 1990. $Ca^{2+}$ influx in human T lymphocytes is induced independently of inositol phosphate production by mobilization of intracellular $Ca^{2+}$ stores. A study with the $Ca^{2+}$ endoplasmic reticulum-ATPase inhibitor thapsigargin. *Eur. J. Immunol*. 20:2269–2275.
29. Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. *Proc. Natl. Acad. Sci. USA*. 87:2466–2470.
30. Telford, W.G., and R.A. Miller. 1996. Detection of plasma membrane Ca(2+)-ATPase activity in mouse T lymphocytes by flow cytometry using fluo-3–loaded vesicles. *Cytometry*. 24:243–250.
31. Berridge, M.J., and R.F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature*. 341:197–205.
32. Parekh, A., A. Fleig, and R. Penner. 1997. The store-operated calcium current I(CRAC): nonlinear activation by InsP3 and dissociation from calcium release. *Cell*. 89:973–980.
33. Weiss, A., G. Koretzky, R. Schatzman, and T. Kadlecek. 1991. Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C-gamma 1. *Proc. Natl. Acad. Sci. USA*. 88:5484–5488.
34. Chan, A.C., T.A. Kadlecek, M.E. Elder, A.H. Filipovich, W.-L. Kuo, M. Iwashima, T.G. Parslow, and A. Weiss. 1994. ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science*. 264:1599–1601.
35. Elder, M.E., D. Lin, J. Clever, A.C. Chan, T.J. Hope, A. Weiss, and T.G. Parslow. 1994. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science*. 264:1596–1599.