Itk Phosphorylation Sites Are Required for Functional Activity in Primary T Cells*

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The Tec family kinase Itk plays a critical role in signal transduction downstream of the T cell antigen receptor and has been implicated in the activation of phospholipase C-γ1, a key regulator of calcium mobilization and extracellular signal-regulated kinase (ERK) activation. We have shown previously that Itk is regulated by an activating transphosphorylation event in which Tyr-511 in the kinase domain is phosphorylated by Lck (Heyeck, S. D., Wilcox, H. M., Bunnell, S. C., and Berg, L. J. (1997) J. Biol. Chem. 272, 25401–25408). In this study, we present evidence for another mode of regulation for Itk, the autophosphorylation of Tyr-180 in the Src homology 3 (SH3) domain. To investigate the role of Itk trans- and autophosphorylation in T cell signaling, a retroviral transduction system was used to introduce different versions of Itk into Itk-deficient primary T cells. We report that Itk mutated at either the trans- or the autophosphorylation site is unable to fully restore cytokine production and ERK activation in the Itk-deficient cells; Itk-Y511F is severely defective, whereas Itk-Y180F has partial activity. Because phosphorylation at Tyr-180 is predicted to interfere with ligand binding by the SH3 domain, an SH3 point mutant that cannot bind ligand was also examined and found to be unable to restore function to the Itk−/− cells. These data provide new insights into the complex regulation of Itk in primary T cells.

Engagement of the T cell antigen receptor (TCR) results in the rapid recruitment and activation of protein tyrosine kinases of the Src, Syk, and Tec families. Genetic and biochemical studies over the last decade have provided a great deal of information about the roles of these kinases in T cell activation, including their mechanisms of regulation and activation and their downstream targets (1). Studies in wild type and mutant Jurkat T cell lines have indicated that the Tec family tyrosine kinase, Itk, is downstream of both Lck and ZAP-70 and is dependent on both; cell lines that lack either Lck or ZAP-70 fail to phosphorylate Itk in response to TCR ligation (2–4). Itk is not a substrate of ZAP-70 but is instead dependent on ZAP-70-mediated LAT phosphorylation for its recruitment to the signaling complex (4, 5). There, Itk serves as a direct substrate of Lck, which activates Itk through phosphorylation of the conserved activation loop tyrosine Tyr-511 (3). A mutant version of Itk that lacks this tyrosine is incapable of either autophosphorylation when expressed in an insect cell expression system, suggesting that phosphorylation of this site is critical to the catalytic activity of Itk.

One of the most important substrates of tyrosine kinases in lymphocytes is phospholipase C-γ1 (PLC-γ1). PLC-γ1 plays a critical role in transmitting signals generated at the cell surface through its hydrolysis of phosphoinositol 4,5-bisphosphate (PIP2) at the cell surface into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). These second messengers mediate the elevation in intracellular calcium and the activation of the Ras-Raf-MEK-ERK pathway that ultimately result in cytokine production and proliferation of the activated T cell. There is now a great deal of evidence that Tec family kinases play a key role in the activation of phospholipase C-γ1 in lymphocytes. In T cells from Itk-deficient mice, stimulation through the TCR results in only low level PLC-γ1 phosphorylation and concomitantly lower calcium mobilization and ERK activation (6, 7). Similar signaling defects have been described in Btk-deficient human, chicken, and mouse B cells (8–10), and mice deficient in PLC-γ2 have a phenotype nearly identical to mice deficient in Btk (11).

Although the functional consequences of an Itk deficiency in T cells has been extensively studied, the mechanisms by which Itk acts in the cell are less well understood. Regulation of the kinase is complex, involving changes in subcellular localization, interactions with other signaling molecules including those of the LAT signaling complex, and an activating transphosphorylation event (reviewed in Ref. 12). Additionally, structural studies have revealed that the protein binding domains of Itk can interact with one another both intra- and intermolecularly (13, 14), suggesting that conformational changes may also modulate the activity of Itk, perhaps by the availability of the protein binding domains of Itk for external interactions.

Another potential mode of regulation for Itk is autophosphorylation. In B cells, transphosphorylation of the Tec kinase Btk by a Src family kinase is followed by Btk autophosphorylation of Tyr-223 in the SH3 domain (15–17). The position of this tyrosine is intriguing as it lies within the substrate binding groove of the SH3 domain, suggesting that phosphorylation at this site may affect protein-protein interactions. A number of groups have assessed the functionality of Btk mutated at Tyr-223, but the role of phosphorylation at this site remains un-
clear. In one study, mutation of Tyr-223 to phenylalanine (Btk-Y223F) potentiated the ability of a Btk variant to transform fibroblasts, suggesting that the mutation had an activating effect on Btk (16). In other systems, though, Btk-Y223F has been found to be partially to severely functionally defective (18, 19).

Tyr-223 in Btk is a conserved residue in many SH3 domains, including that of Itk, although it has not been identified as a site of phosphorylation in other families. Based on structural studies, phosphorylation at this site is predicted to be incompatible with SH3 ligand binding in either Itk or Btk (13, 20). For this reason, the effect of phosphorylation on protein function is likely dependent on the role played by the SH3 domain. In the case of Btk and Itk, important differences have been described in both the structures and modes of regulation of the two kinases in this region. The SH3 domains of Itk and Btk have only 51% sequence identity, and it has been reported that the Btk SH3 backbone conformation is more similar to that of Fyn or c-Src than to that of Itk (20). Furthermore, structural analyses of the adjacent Tec homology (TH) and SH3 regions of Btk and Itk indicate that the two proteins self-associate in distinct intramolecular and intermolecular conformations, suggesting unique modes of regulation (13, 20–22).

In the present study, we use an insect cell expression system to demonstrate that Lck-activated Itk autophosphorylates at Tyr-180 in the SH3 domain, homologous to Btk Tyr-223. Mutation of this site results in a catalytically active and capable of both auto- and transphosphorylation site, results in a protein incapable of phosphorylating PLC-γ1 in this system. To evaluate the effects of Itk trans- and autophosphorylation events in T cell signaling, we developed a system in which variants of Itk are introduced into Itk-deficient primary CD4+ T cells using a retroviral vector, and the functional consequences assessed. We show that the transphosphorylation site in Itk, Tyr-511, is essential for the activity of the protein in primary T cells, as expression of Itk-Y511F in Itk−/− T cells does not restore functionality to the cells. Expression of Itk mutated at the autophosphorylation site, Tyr-180, results in a partial restoration of the signaling pathways defective in the mutant cells, indicating that this site plays a non-essential but positive role in Itk signaling. Finally, to assess the role of the SH3 domain in Itk signaling, we examined a second SH3 domain point mutant, Itk-W208K, that is unable to bind proline motif-containing ligands in vitro (23). In this study we show that Itk-W208K is catalytically active and capable of both auto- and transphosphorylation when expressed in insect cells. However, this mutant is severely defective when expressed in vivo and is unable to functionally reconstitute Itk−/− T cells.

EXPERIMENTAL PROCEDURES

SP/Y Insect Cell Baculovirus Expression System—SP/Y insect cells were obtained from Pharmingen and grown in TMN medium (Pharmingen) as adherent cultures. Baculoviruses encoding Itk, Lck, IκB, and IκB-ΔN11F have been described previously (5). Itk-Y180F and Itk-W208K were generated by site-directed mutagenesis and subcloned into the Fastbac1 vector (Invitrogen). cDNA encoding rat PLC-γ1 in a pCDNA3.1/Zeo (+) vector was a gift of Dr. Sue Goo Rhee (National Institutes of Health). The gene was excised as a BamHI fragment and cloned into Fastbac1. Cells were infected, harvested, and lysed as described previously (3).

Immuno precipitation, Immunoblotting, and Immune Complex Kinase Assays—Immunoprecipitation and immunoblotting were performed as described previously (24). Anti-Itk mAbs 2F12 and 10B2 were generated as described previously (3). A combination of five anti-PLC-γ1 mAbs was purchased from Upstate Biotech, Inc. Anti-phosphotyrosine (4G10) and anti-pY783 PLC-γ1 polyclonal antibody was purchased from Biosource. The anti-Btk phosphospecific antibodies 4F10 and 8D11 were a kind gift of Dr. Matthew Wahl and Dr. Owen Witte of UCLA. Anti-phosphotyrosine antibody 4G10 was a kind gift of Dr. Brian Druker of Oregon Health & Science University. Anti-phospho-ERK1/2 and anti-ERK1/2 rabbit polyclonal antibodies were purchased from Cell Signaling Technology.

For in vitro immune complex kinase assays, Itk samples were immunoprecipitated and washed several times in lysis buffer, followed by a rinse in wash buffer (20 mM Tris-Cl, pH 7.4, and 1 mM sodium vanadate). Immunoprecipitates were then incubated at 37 °C for 25 min in kinase assay buffer (20 mM Tris-Cl, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, 20 μM cold rATP, and 12.5 μM of γ-32PATP). Reactions were stopped by the addition of SDS loading dye, and the samples were boiled and loaded onto SDS gels. Proteins were transferred to polyvinylidene fluoride membrane (Millipore) and either exposed to film overnight for 32P detection or subjected to immunoblot analysis.

Mice—Itk-deficient mice were made by targeted gene disruption (6). The mice were backcrossed (nine generations) onto a C57BL6 background. A subset of experiments was performed on incompletely backcrossed mice whose genetic background was a mix of 120 and C57BL6, and the results of these experiments were indistinguishable from those performed on more completely backcrossed mice. Mice were housed in specific pathogen-free (SPF) facilities and sacrificed for cell harvesting between six and eight weeks of age.

Cell Fractionation and Expansion—Lymph nodes were isolated from 6- to 8-week-old C57BL6 mice and homogenized. Lymph node samples were separated from stroma by filtration and washed multiple times in RPMI 1640 medium supplemented with HEPES and 3% fetal calf serum. CD8+ T cells were removed by complement-mediated lysis (anti-CD8 IgM mAb ascites, clone 3.155, and rabbit complement; Cedarlane) followed by removal of dead cells and red blood cells using a Lympholyte-M (Cedarlane) density gradient. Cells were then washed multiple times and stimulated in complete RPMI-10 medium (RPMI 1640, 10% fetal calf serum, HEPES, pen-strep, glibutamine, and β-mercaptoethanol) supplemented with 5 ng/ml phorbol myristate acetate (PMA) and 0.375 mM ionomycin at 5 × 105 cells/ml. After 24–48 h, cells were spun down and resuspended in complete RPMI-10 supplemented with 5 ng/ml recombinant IL-2 (Amgen). Cells were then cultured an additional 8–12 days, and cell density was maintained between 5 × 105 and 2 × 106 cells/ml. Experiments were performed between days 10 and 14 following stimulation. For some experiments, cells were restimulated between days 12 and 14 with PMA and ionomycin (as before) and expanded for an additional 8–12 days prior to their use.

Retrovirus Production—The retroviral mouse stem cell virus vector, MSCV2.2-IRES-GFP, was a gift from Bill Sha (University of California at Berkeley) and has been described previously (25, 26). Itk and Itk variants were cloned into the vector as NotI or Cla1 fragments and screened for correct orientation. The Phoenix-E retroviral packaging cell line was obtained from American Type Culture Collection (ATCC), and Phoenix-E cells were grown in complete RPMI-10 medium. Supernatants, 2 × 106 cells were plated per each 100-mm culture dish 8–14 h prior to transfection (~50% confluence). Transfections were performed using FuGENE-6 transfection reagent (Roche Applied Science). The manufacturer’s protocol was used but was scaled for use in 100-mm plates as follows. 10 μg of DNA and 15 μl of FuGENE were used per plate, and the mixture was added to the plates in 500 μl of Opti-MEM serum-free medium (Invitrogen). After 6–14 h in transfection medium, the supernatant was aspirated and replaced with complete RPMI-10 (6 ml/plate). ~24 h after initiation of transfection, the plates were moved from a 37 °C incubator to a 32 °C incubator to minimize the breakdown of the produced virus. Viral supernatants were collected every 48 h and replaced with fresh medium. Supernatants were harvested repeatedly until the cells died (typically four to five harvests). After collection, supernatants from duplicate plates were pooled, spun down to remove cell debris, quick frozen in liquid N2, and stored at −80 °C. Transfection rates of the Phoenix-E cells were typically 65–90%.

Retroviral Infection and Expansion of T Cell Lines—CD4+ T cell lines were harvested 48 h after transfection, the cells were collected, counted, and aliquoted for infection with different viruses. Generally, 106 cells were infected with each virus. In each experiment, samples of Itk−/− and Itk−/− cells were left uninfected or infected with virus containing vector alone (GFP, but no Itk gene). A sample of Itk−/− cells infected with wild type Itk virus was always included as a control. Viral supernatants were harvested immediately before use. To infect 106 cells, the following viral mixture was used: 2 ml of viral supernatant, 60 μl of fetal calf serum, 40 μl of IL-2 supernatant, and 16 μg of Lipofectin reagent (Invitrogen). The mixture was mixed thoroughly and allowed to sit at room temperature for 60 min.
before it was added to cells. Cells were resuspended in the mixture, plated out in 6-well dishes (35 mm), spun at 2000 rpm for 45 min at 25 °C, and incubated for 6 h to overnight at 37 °C. After this period, the cells were collected, spun down, and the infection repeated with fresh viral mixture. After an additional 6 h to overnight, the cells were collected, spun down, and resuspended at 5 × 10^6/ml in normal IL-2-containing medium.

Cell Sorting for CD4+ GFP+ Cells—3–4 days after retroviral infection, cultured cells were collected, stained with anti-CD4-PE (Pharmingen), and sorted for GFP and CD4 on a FACS Vantage (Flow Cytometry Facility, University of Massachusetts Medical School). Typically, cells were 85–95% CD4+ and 10–5% GFP+ prior to sorting. CD4+ GFP+ cells were grown in IL-2 medium as done previously, and functional assays were performed on days 10–12 post-harvest.

IL-2 Production and ERK Activation Assays—For IL-2 assays, round-bottom, 96-well plates were coated with 5 μg/ml goat anti-hamster antibody (Roche Applied Science) in carbonate buffer (pH 9.5) followed by a mix of anti-CD3 (clone 2C11) and anti-trinitrophenyl (isotype-matched control) antibodies (Pharmingen). The total concentration of hamster antibody added to each well was constant (3 μg/ml, unless otherwise noted) with the concentration of anti-CD3 antibody as noted in the legends to Figs. 3–6. Cultured CD4+ cells were stimulated and analyzed for IL-2 production as described (27), with the exception that monensin was not included in the stimulation medium and that 5 μg/ml brefeldin A was substituted for monensin in the staining buffer. Cells were analyzed by flow cytometry immediately after staining. Samples from each population were checked for CD4 and GFP expression and were consistently >95% positive for each marker. IL-2 dot plots are presented using an arbitrary linear channel as the x-axis. For ERK activation assays, cultured CD4+ T cells were stimulated and analyzed as described (7). The lysis buffer used was the same as that described above for Sf9 cells, except for the addition of 0.3% deoxycholate (DOC). Cells were lysed at a density of 10^6 cell equivalents/ml, and protein content of the cleared lysates was determined by Bradford analysis.

RESULTS

Biochemical Analysis of Itk Phosphorylation Sites—To investigate the roles of tyrosine phosphorylation in Itk, we used a baculovirus system to express recombinant proteins in Sf9 insect cells. Baculoviruses encoding wild type Itk, kinase-inactive Itk-K390R, and Itk with tyrosine-to-phenylalanine substitutions at either the site of transphosphorylation, Tyr-511, or the candidate site of autophosphorylation, Tyr-180, were generated (Fig. 1A). In addition, Itk-W208K, a point mutant in which SH3 ligand binding is abolished, was also included.

To investigate the phosphorylation states of these mutant versions of Itk, the proteins were expressed in Sf9 insect cells, either with or without coexpression of Lck. Itk was then immunoprecipitated from cell lysates and blotted for phosphotyrosine content. Three different anti-phosphotyrosine mAbs were used to probe the membrane, i.e. 4G10, an antibody that recognizes total phosphotyrosine, and two anti-Btk phosphospecific antibodies, namely 8D11, raised against Btk phosphorylated at Tyr-551 (homologous to Tyr-511 in Itk), and 4F10, raised against Btk phosphorylated at Tyr-223 (homologous to Tyr-180 in Itk) (28). For clarity, the antibodies 8D11 and 4F10 will hereafter be referred to as anti-pY511 and anti-pY180, respectively.

As we have reported previously, Lck transphosphorylates Itk at Tyr-511, and the presence of this tyrosine is critical for the catalytic activity of Itk (3). These findings are also illustrated in Fig. 1B. Coexpression of Lck with either wild type or kinase-inactive Itk results in Itk tyrosine phosphorylation (Fig. 1B, lanes 4 and 6, 4G10 blot). Because this phosphorylation does not require the catalytic activity of Itk, we conclude that Itk is directly phosphorylated by Lck. This transphosphorylation occurs at Tyr-511, as reported previously and as confirmed here by blotting with an anti-phosphospecific antibody (pY511 blot) (3). This finding is also confirmed by analysis of the mutant Itk-Y511F, which is substantially less phosphorylated by Lck than either wild type Itk or Itk-K390R (Fig. 1B, lane 12, 4G10 blot).

Phosphorylation of Itk by Lck results in increased Itk catalytic activity. Activation-induced autophosphorylation can be seen in Fig. 1B by comparing the phosphotyrosine content of Itk in the wild type (WT) plus Lck and K390R plus Lck samples. Although the two samples have similar levels of phosphorylation at Tyr-511 (lanes 4 and 6, pY511 blot), only wild type Itk is capable of autophosphorylating, resulting in greater total phosphotyrosine (4G10 blot). The mutant Itk-Y511F cannot be activated by Lck and therefore fails to autophosphorylate (lane 12, 4G10 blot).

To determine whether autophosphorylation of Itk occurs at the candidate site, Tyr-180, the mutant Itk-Y180F was analyzed. When expressed alone, this mutant shows moderately reduced basal phosphorylation relative to wild type Itk (data not shown). When coexpressed with Lck, Itk-Y180F becomes phosphorylated as indicated by immunoblotting with either the 4G10 or the pY511 antibody (Fig. 1B, lane 10). When probed with the pY180 antibody, Lck-activated wild type Itk shows significant reactivity (lane 4, pY180 blot). In contrast, Itk-Y180F, which shows high levels of total phosphorylation by 4G10 blot, shows no reactivity when probed with pY180 (lane 10, pY180 blot). These data confirm the specificity of the pY180 antibody and identify Tyr-180 as a site of phosphorylation. The SH3 binding mutant, Itk-W208K, was also analyzed for its ability to become trans- and autophosphorylated. Fig. 1B shows that this mutant is similar to wild type in its phosphorylation pattern.

The data also demonstrate that Tyr-180 is a site of autophosphorylation. A, schematic representation of the domain structure of Itk, with positions of point mutations indicated. The mutant Itk-W208K has a C-terminal Myc tag, causing it to migrate slightly slower than other species on SDS gels. B, Sf9 cells were infected with baculovirus expressing the indicated version of Itk, either with (+) or without (−) coexpression of the Lck virus. Itk was immunoprecipitated (IP) from cell lysates and analyzed for phosphotyrosine content by sequential immunoblotting (BLOT) with anti-phosphotyrosine antibody 4G10 (pY), anti-phosphopeptide specific antibody 8D11 (pY511), and anti-phosphopeptide specific antibody 4F10 (pY180). Itk levels were similar in each of the immunoprecipitates, as indicated by reprobing the membrane with anti-Itk antibody. It should be noted that longer exposure of the 4G10 blot reveals low level autophosphorylation of WT Itk and Itk-W208K, and, to a lesser extent, Itk-Y180F in the absence of Lck, as has been demonstrated previously with this system (3). Data shown are representative of four separate experiments.
phorylation rather than a second site of Lck phosphorylation. When coexpressed with Lck, wild type Itk becomes phosphorylated at both Tyr-511 and Tyr-180 (Fig. 1B, lane 4, pY511 and pY180 blots). In contrast, Itk-K390R becomes phosphorylated only at Tyr-511, indicating that the kinase activity of Itk is required for phosphorylation at Tyr-180. Together, these data demonstrate that Tyr-180 in Itk is a site of activation-induced autophosphorylation. Lck greatly enhances phosphorylation at this site but is incapable of directly phosphorylating it. These data strongly support a model in which transphosphorylation of Itk by Lck at Tyr-511 induces the catalytic activity of Itk, resulting in autophosphorylation of Tyr-180 in the SH3 domain. These findings are in agreement with studies on Btk, which indicate that phosphorylation of Tyr-551 by a Src family kinase precedes Btk autophosphorylation of Tyr-223 (16, 17, 28, 29).

In studies using Jurkat T cells, Itk has been shown to become phosphorylated following CD3 crosslinking in an Lck-dependent manner (3, 30). To directly assess whether Itk Tyr-511 and Tyr-180 become phosphorylated in T cells, Itk immunoprecipitates from resting or stimulated Jurkat cells were probed with the anti-phosphospecific antibodies. Immunoblotting with anti-pY511 showed that Itk becomes inducibly phosphorylated at this site in a pattern identical to that seen when the membrane was probed with 4G10 (data not shown). However, we were unable to detect reactivity using the antibody pY180. We believe that this may reflect a relatively low affinity of the antibody for Itk-pY180, perhaps due to weak conservation between the Btk epitope and the homologous region of Itk (40% sequence identity). It is also possible that phosphorylation at this site occurs only at low stoichiometry in activated T cells and is thus below the limits of detection.

**Functional Analysis of Itk Mutants in Vitro and in Vivo in Insect Cells**—We next sought to assess the functional consequences of autophosphorylation at Tyr-180 by measuring the catalytic activity of the mutant Itk-Y180F. Itk was immunoprecipitated from SF9 cells infected with either wild type or mutant versions of Itk and subjected to an *in vitro* autophosphorylation kinase assay. As shown in Fig. 2A, the mutant Itk-Y180F is capable of autophosphorylating, indicating that phosphorylation at Tyr-180 is not required for the catalytic activity of Itk. The results further suggest that there is at least one other tyrosine that Itk is capable of autophosphorylation in vitro, although it is not known if this phosphorylation occurs *in vivo*. The SH3 binding mutant, Itk-W208K, has normal catalytic activity, and Itk-Y511F, as reported previously, has diminished catalytic activity relative to wild type Itk, although it is consistently higher than that of Itk-K390R (3).

We next examined the ability of Itk-Y180F and the other mutants to phosphorylate substrates *in vivo* in the insect cells. First, total lysates from infected insect cells were examined for the presence of tyrosine-phosphorylated proteins. As seen in Fig. 2B, wild type Itk, Itk-W208K, and Itk-Y180F tyrosine phosphorylate a variety of insect cell proteins, whereas Itk-K390R and Itk-Y511F do not. It should be noted that overexpression of Itk in this system results in an active protein, even in the absence of Lck. This activity is not phosphorylation-independent, as indicated by the inactivity of the mutant Itk-Y511F. Instead, we believe that, under such overexpression conditions, Itk has some capacity to autophosphorylate at Tyr-511, though this is unlikely to occur in T cells. For the purposes of the experiments shown in Fig. 2, we have excluded Lck to avoid the complication of the presence of two different kinases.

We next examined the ability of wild type or mutant Itk proteins to phosphorylate the Tec family substrate PLC-γ1. SF9 cells were coinfected with PLC-γ1 and each of the Itk mutants indicated. PLC-γ1 was then immunoprecipitated and analyzed for phosphotyrosine content by sequentially blotting with 4G10 and PLC-γ1-pY783, an anti-phosphospecific antibody that specifically recognizes PLC-γ1 phosphorylated at Tyr-783. Phosphorylation at this site is thought to be critical for the regulation of PLC-γ1 (31). We show that PLC-γ1 expressed alone or in the presence of the mutants Itk-K390R or Itk-Y511F does not become phosphorylated (Fig. 2C). In contrast, coexpression of PLC-γ1 with wild type Itk, Itk-W208K, or Itk-Y180F results in PLC-γ1 tyrosine phosphorylation. These data conclusively demonstrate that Itk is capable of directly phosphorylating PLC-γ1, and further, that mutation of the autophosphorylation

**Fig. 2.** Itk-Y180F is catalytically active in cells. SF9 insect cells were infected with baculoviruses expressing the indicated versions of Itk. A, Itk was immunoprecipitated from cell lysates, subjected to an in *vitro* immune complex kinase assay (IVK), separated on an SDS-PAGE gel, and transferred to nitrocellulose. Incorporation of 32P was detected by exposure to radiographic film (top section). Itk levels were similar in all immunoprecipitates, as indicated by immunoblot (bottom section). B, phosphorylation of cellular proteins was analyzed by immunoblotting lysates of infected cells with anti-phosphotyrosine (4G10). Itk levels in each of the lysates were similar, as indicated by immunoblot with anti-Itk antibody. C, cells infected with different versions of Itk were cotransfected with a baculovirus expressing PLC-γ1. PLC-γ1 was immunoprecipitated (IP) and analyzed for phosphotyrosine content by sequential immunoblotting with anti-phosphotyrosine antibody 4G10 (pY) (top section) and anti-pY783 PLC-γ1 antibody (middle section). Levels of PLC-γ1 in each of the immunoprecipitates were similar, as indicated by blotting with anti-PLC-γ1 antibody (bottom section).
site Tyr-180 does not significantly affect the ability of Itk to phosphorylate substrates in a heterologous overexpression system.

It should be noted that our data do not preclude the existence of additional phosphorylation sites in Itk beyond Tyr-180 and Tyr-511. Indeed, the insect cell data suggest that there may be additional sites of both auto- and transphosphorylation. Analyses of a double mutant, Itk-Y180F/Y511F, further support this conclusion in that the residual Lck phosphorylation and kinase assay activity observed with Itk-Y511F are also observed with the double mutant (data not shown).

**Reconstitution of Itk Protein Expression in Primary Itk−/− T Cells**—Insect cells provide a valuable system in which to study protein biochemistry. However, they lack the many regulatory and signal transducing molecules found in lymphocytes, with which Itk normally interacts. To evaluate the Itk phosphorylation site mutants in a more physiological setting, we established a system in which wild type or mutant Itk genes could be introduced into Itk-deficient primary T cells by using a retroviral vector that also carries the GFP gene. Productive retroviral infection requires that cells be actively cycling, so lymphocytes isolated directly from a mouse must be activated prior to gene transfer. Because the Itk-deficient T cells have a defect in signal transduction downstream of the TCR/CD3 complex, the pharmacological agents PMA and ionomycin were used to stimulate ex vivo CD4+ T cells, and exogenous IL-2 was provided to maximize cell proliferation. Under these stimulation and culture conditions, Itk+/+ and Itk−/− CD4+ T cells exhibit similar kinetics with regards to growth rate and expression of activation markers (data not shown). After retroviral infection and expansion, the cells were sorted for GFP+ cells, and functional assays were performed upon secondary stimulation of the cultured, retrovirally infected cells.

For this gene transfer system to be useful, it was necessary to ensure the following: 1) that the cultured cell lines maintain the functional defects seen with freshly isolated Itk and Itk−/− T cells; and 2) that reconstitution of the cells with retrovirally introduced wild type Itk restores IL-2 production. These conditions were met, as shown in Fig. 3A. In this experiment, IL-2 production was compared among three cell populations, i.e. Itk−/− T cells infected with vector alone, Itk−/− T cells infected with vector alone, or Itk−/− T cells infected with the virus encoding wild type Itk. The cultures were grown and sorted for infected cells as described previously. After two rounds of PMA/ionomycin stimulation and expansion, the cells were restimulated with plate-bound anti-CD3 and analyzed for cytokine production in an intracellular IL-2 flow cytometry assay.

Comparing the left and center panels of Fig. 3A, it is clear that IL-2 is produced by fewer Itk−/− T cells than Itk+/+ T cells in response to CD3 crosslinking (35% versus 61%). It is also apparent that, among cells that do produce IL-2, the average amount produced per cell is significantly lower in the Itk−/− population than in the control population, as indicated by the difference in the mean fluorescence of IL-2-producing cells in each group (Fig. 3A). These results suggest that the diminished capacity of Itk−/− T cells to produce IL-2 has two causes, i.e. fewer cells reach the required signaling threshold to initiate IL-2 production, and, of those that do, relatively low amounts of IL-2 are synthesized per cell. Analysis of total IL-2 production by an enzyme-linked immunosorbent assay (ELISA) or an IL-2 bioassay reveals that Itk−/− cells secrete at least 10-fold less IL-2 than do wild type cells (Ref. 6, and data not shown). Together, these data suggest that the paucity of IL-2 “high producers” in the Itk−/− T cell population results in a profound reduction in the total IL-2 produced and secreted.

The third panel from the right in Fig. 3A shows the IL-2 produced in Itk−/− T cells reconstituted with wild type Itk. Over a wide range of anti-CD3 antibody concentrations (0.01–5 μg/ml), Itk−/− T cells reconstituted with wild type Itk produce IL-2 at levels equal to or exceeding that of Itk+/+ T cells (data not shown). Using the intracellular cytokine staining assay, we find that both the percentage of responding cells and the average amount of IL-2 synthesized per cell are restored to wild type levels in the reconstituted Itk−/− T cells.

The levels of Itk expressed in retrovirally infected cells cannot be easily manipulated in this system. Infection with viral stocks of differing titers results in a greater or lesser number of cells being infected but does not affect the levels of protein expressed per cell. To determine how Itk levels in retrovirally infected Itk−/− T cells compare with the endogenous levels of Itk found in wild type T cells, Itk was immunoprecipitated from lysates of Itk+/+ or from Itk−/− cells with or without retrovirally expressed wild type Itk. As shown in Fig. 3B, the amount of Itk expressed in the infected Itk−/− T cells is 10-fold lower than that of endogenous Itk in Itk+/+ T cells (comparing immunoprecipitations from 105 infected Itk−/− cells to that of 106 Itk+/+ cells). Interestingly, this low level of Itk completely restores IL-2 production by the Itk−/− T cells. Based on this finding, we decided that the most appropriate positive
control for our analysis of Itk mutants was Itk−/− cells reconstituted with wild type Itk.

To represent the IL-2 data graphically, the responses of Itk−/− T cells reconstituted with vector alone or with a given Itk mutant are normalized to the response of cells reconstituted with wild type Itk. A representative conversion from flow cytometric data (Fig. 4A) to graph (Fig. 4B) is shown in Fig. 4. In this experiment, cultured Itk−/− cells reconstituted with either vector alone or wild type Itk were stimulated with a range of concentrations of plate-bound anti-CD3 antibody and analyzed for IL-2 production (Fig. 4B). It is interesting to note that, as cells are stimulated with limiting amounts of anti-CD3 antibody, the difference between cells that do or do not express Itk becomes more pronounced (Fig. 4B). The same trend is seen in a comparison between Itk−/− and Itk−/− T cells (data not shown). These findings suggest that the presence of Itk might be particularly critical under conditions in which antigen is limiting.

**Functional Analysis of Itk Mutants in Primary T Cells**—We next compared Itk−/− T cells reconstituted with wild type or mutant versions of Itk. Fig. 5A shows the GFP profiles of retrovirally infected cells from a representative experiment. We observe routinely that cells infected with vector alone express higher levels of GFP than cells infected with Itk-containing constructs. Importantly though, among cells expressing different Itk mutants, GFP expression levels are uniform, both within and between experiments. Because the Itk and GFP genes are translated from a single RNA transcript, the homogeneity of the GFP profiles indicates that wild type and mutant Itk proteins should be expressed at the same level. This expectation is confirmed by immunoblotting lysates of reconstituted cells with an Itk specific antibody (Fig. 5B, and data not shown).

In Fig. 5C, the ability of cells expressing different versions of Itk to produce IL-2 is depicted. The data was compiled from multiple independent experiments; in each case, the response of cells expressing vector or an Itk mutant was normalized to the response of cells reconstituted with wild type Itk in the same experiment. This normalized data was then averaged with that of other experiments and graphed as in Fig. 4. The **top panel** of Fig. 5C compares IL-2 production in CD3-stimulated cells expressing either vector, Itk-K390R, Itk-Y511F, or wild type Itk. Expression of either Itk-K390R or Itk-Y511F fails to restore IL-2 production in the Itk−/− T cells. These data clearly establish that the enzymatic activity of Itk is required for its function in the potentiation of IL-2 production and, furthermore, that phosphorylation of Tyr-511, presumably by a Src family kinase, is required for this activity.

The **bottom panel** of Fig. 5C compares cells expressing either vector, Itk-Y180F, Itk-W208K, or wild type Itk. Expression of Itk-Y180F results in a partial restoration of the cells’ ability to produce IL-2 in response to TCR stimulation. At the higher concentrations of anti-CD3 antibody, Itk-Y180F restores IL-2 production nearly as well as does wild type Itk. However, under sub-optimal stimulation conditions, cells expressing Itk-Y180F are substantially less effective at inducing IL-2 synthesis than are those expressing wild type Itk. Because phosphorylation of Tyr-180 is predicted to interfere with SH3 domain ligand binding, we also examined IL-2 production in cells reconstituted with the SH3 binding mutant, Itk-W208K. Although this point mutation abolishes ligand binding in vitro, an Itk protein carrying this mutation has normal catalytic activity when expressed in insect cells. In reconstituted Itk T cells, we found that Itk-W208K is incapable of restoring IL-2 production (Fig. 5C, bottom panel). Together, these data indicate that SH3 domain ligand binding is essential for the function of Itk in T cells and that the presence of Tyr-180 is required for optimal activity.

**Upstream Signaling Events**—One of the primary defects in Itk T cells is their inability to fully activate PLC-γ1. Restoration of PLC-γ1 activation is the likely mechanism by which cytokine production is restored in Itk−/− cells reconstituted with wild type Itk. We were unable to directly assess changes in PLC-γ1 phosphorylation or activation because of the limiting cell numbers attainable in the reconstitution system. Instead, we investigated a downstream event, the phosphorylation of ERK1 and ERK2 in response to CD3 crosslinking (32, 33). Previous studies have shown that ERK phosphorylation is impaired in Itk−/− T cells (6, 7), and the cultured cells described in this study retain this defect.

To assess the ability of wild type and mutant Itk to restore ERK phosphorylation in reconstituted cells, Itk−/− and Itk−/− cells were stimulated and grown as described previously. Cultures of Itk−/− T cells were infected with retroviruses encoding wild type or mutant Itk, sorted and expanded, and then stimulated by anti-CD3 crosslinking for the time periods indicated.
As shown in Fig. 6, ERK phosphorylation is impaired in cell lines lacking Itk. This defect is fully corrected in cells retrovirally reconstituted with wild type Itk. In contrast, expression of the catalytically inactive mutant Itk-K390R does not restore ERK phosphorylation in response to TCR stimulation. Expression of Itk-Y511F also fails to restore ERK phosphorylation in the Itk⁻/⁻ T cells, confirming that this mutant is functionally inactive. Expression of Itk-Y180F results in partial restoration of ERK activation, consistent with the intermediate restoration of IL-2 production by this mutant. Finally, preliminary data indicates that the mutant Itk-W208K is severely impaired in its ability to restore ERK activation to Itk⁻/⁻ cells. Thus, the ability of wild type or mutant Itk to restore ERK phosphorylation correlates well with the effect of each on IL-2 production.

CONCLUSIONS

In the present study, we identify Tyr-180 in the Itk SH3 domain as a site of activation-induced autophosphorylation and investigate the role of phosphorylation sites in the function of Itk. Our data strongly support a model in which transphosphorylation of the activation loop tyrosine precedes (and is required for) Itk autophosphorylation in the SH3 domain. This model is based on studies of recombinant proteins expressed in insect cells, which indicate that the Src-family kinase, Lck, provides the initial activating phosphorylation event at Tyr-511, stimulating Itk to autophosphorylate at Tyr-180. Further support for such a "two-step" activation model comes from similar studies on the related kinase, Btk. Several studies have shown that both Tyr-551 and Tyr-223 in Btk (analogous to Tyr-511 and Tyr-180, respectively, in Itk) become phosphorylated following ligation of the B cell antigen receptor (BCR) and

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2 H. M. Wilcox and L. J. Berg, manuscript in preparation.
that the kinetics of these phosphorylation events support a model in which phosphorylation at position Tyr-551 precedes that at Tyr-223 (16, 17, 28, 29).

Itk mutants with substitutions at these conserved phosphorylation sites were also examined for their catalytic activity in the insect cell system. The activation loop mutant, Itk-Y511F, was found to be severely defective in both autophosphorylation and phosphorylation of cellular proteins or the coexpressed substrate, PLC-γ1. These findings are in agreement with those of numerous studies in both lymphocytes and heterologous expression systems that show that both Btk-Y551 and Itk-Y511 are critical for the catalytic activity of these kinases, reflecting the important role of Src family-mediated phosphorylation for Tec family activation (29, 34). In contrast, Itk-Y180F showed no defects in these assays, indicating that autophosphorylation in the SH3 domain is not required for catalytic activity in this system.

As discussed previously, autophosphorylation in the SH3 domains of Itk and Btk may not have the same functional consequences for each protein. Nevertheless, studies of Btk-Y223F are illustrative in that they demonstrate that the apparent importance of this site can vary significantly, depending on the systems and assays used. For example, Park et al. found that mutating Btk-Y223 to phenylalanine potentiated the ability of a gain-of-function Btk mutant to transform fibroblasts, suggesting that phosphorylation of Btk-Y223 might negatively regulate the protein’s catalytic activity (16). However, the same study showed that Btk-Y223F immunoprecipitated from these cells did not have elevated kinase activity in vitro but was similar to wild type Btk in its ability to both autophosphorylate and transphosphorylate enolase. Other studies have shown moderate to severe defects in the function of Btk-Y223F. In one study, Btk-Y223F expressed in 293 cells was unable to phosphorylate the coexpressed substrate BAP-135, suggesting that the mutant was functionally defective in these cells (19). Another group expressed Btk-Y223F in a transformed Btk-deficient chicken cell line and found that it was partially able to restore calcium mobilization, IP3 production, and PLC-γ2 phosphorylation (18).

One difficulty in evaluating Tec family kinases in heterologous systems is that their activation is normally dependent on a complex series of interactions with upstream regulators and binding partners, many of which are lymphocyte-specific. It is possible that the physiological effect of Btk or Itk autophosphorylation is to modulate SH3 domain interactions with lymphocyte-specific signaling molecules, in which case studies in non-lymphoid cells may not be sufficient to elucidate the function of this phosphorylation event. Studies in lymphoid systems may be preferable for this reason but can have other limitations. Many methods for introducing ectopic genes into cells result in high levels of protein expression, and overexpression of Tec family kinases in lymphocytes can have effects on antigen receptor signaling paradoxically similar to those seen in kinase-deficient cells. For example, B cells from mice that over-express Btk by 50% showed reduced proliferative responses to B cell antigen receptor cross-linking and type II T-independent antigens (35, 36). Similarly, overexpression of Itk in Jurkat cells was shown to antagonize nuclear factor of activated T cells (NFAT)-mediated transcription (23) despite the genetic evidence that a deficiency in Itk also results in decreased NFAT activity (37). These data suggest that overexpression of Tec kinases disrupts the stoichiometry of signaling molecules that must interact precisely to transmit a downstream signal. It has been postulated that the transcriptional suppression seen in Jurkat cells overexpressing Itk is a consequence of competition between Itk and other signaling molecules for limited numbers of docking sites on the LAT/SLP-76 complex (23). Studies of Itk mutants in Jurkat cells are further complicated by the presence of the endogenous Itk proteins and by the dysregulation of lipid metabolism in these cells, which causes Itk to be constitutively associated with the plasma membrane (38).

To avoid some of these difficulties, we adopted a retroviral transduction system to introduce Itk mutants into primary T cells lacking endogenous Itk. The Itk−/− cells have defects in ERK activation and IL-2 production that are fully corrected by the ectopic expression of wild type Itk. Surprisingly, restoration is achieved by the expression of relatively low levels of wild type Itk compared with the endogenous levels of Itk in wild type cells. The explanation for this effect is not known. Thymic development is altered in Itk−/− mice (39–41), and it is possible that T cells found in the peripheral organs of such mice have made compensatory changes that allowed them to develop and survive. Primary Itk−/− T cells have been analyzed for regulation of the TCR family members Tec and Rlk but appear to have normal levels of these proteins (6). For the purposes of our studies, the complete restoration of IL-2 production and ERK phosphorylation in Itk−/− cells reconstituted with wild type Itk provides a meaningful system in which to assess the relative abilities of different Itk mutants to functionally restore TCR signaling.

Using the retroviral system, we investigated the ability of each of the phosphorylation site mutants to reconstitute TCR signaling in Itk−/− T cells. In contrast to the functional restoration achieved by wild type Itk, neither of the Itk phosphorylation site mutants was able to fully correct the signaling defects of Itk−/− cells. Itk-Y511F was severely defective; cells expressing this mutant were indistinguishable from cells expressing a catalytically inactive mutant, Itk-K390R, or from cells expressing the empty retroviral vector. The complete loss of activity resulting from a mutation at Tyr-511 fits well with previously reported in vitro data indicating that this mutation ablates the catalytic activity of the protein (3). The autophosphorylation site mutant Itk-Y180F had intermediate activity, partially restoring both ERK phosphorylation and IL-2 production. These data demonstrate that both phosphorylation sites are required for optimal Itk activity in primary T cells, though only Tyr-511 is essential.

3 A. T. Miller and L. J. Berg, unpublished observations.
We also investigated the ability of the mutant Itk-W208K to restore function to Itk-/- T cells and found that disruption of the ligand binding capacity of the SH3 domain in Itk severely diminished the functionality of the protein. It should be noted that these results do not conflict with the recent finding that deletion of the SH3 domain in Itk (ItkΔSH3) results in increased basal activity in a fibroblast expression system (42). In our own studies, it is likely that the failure of the Itk-W208K mutant to restore IL-2 production to Itk-/- T cells is due to its inability to form protein-protein interactions with other signaling components in the T cells rather than to a direct impact on the catalytic activity. The findings of Hao and August (42) may reflect structural changes in ItkΔSH3 that lead to altered kinase activity in a non-TCR dependent system.

Regulation of Itk by autophosphorylation is not completely understood. However, our finding that the SH3 domain has an essential positive role in signaling limits the possible interpretations. Structural studies have indicated that phosphorylation at Tyr-180 is likely to interfere with SH3 domain ligand binding because of the addition of a bulky, negatively charged moiety in the binding cleft (13, 43). Given that SH3 binding plays a positive role, the simplest model predicts that mutating Tyr-180 would result in increased responsiveness to TCR signaling because of the alleviation of negative regulation of the SH3 domain. Instead, we found that the ability of the SH3 domain to bind ligand was essential for the function of Itk, and, yet, mutating Tyr-180 resulted in a moderate reduction in activity.

Our interpretation of these data is that the SH3 domain plays both positive and negative regulatory roles in the activation of Itk and that these roles are temporally regulated. Itk activation is a complex, multistep process, and it is probable that some domains have multiple, temporally distinct roles. We believe that the SH3 domain plays an essential, positive role early in the activation sequence of Itk, perhaps through its interactions with SLP-76, the adaptor molecule thought to link Itk to LAT (23, 44, 45). Phosphorylation of Tyr-180 can occur early in Itk activation, and the subsequent phosphorylation of Tyr-180 prolongs signaling by interfering with a later SH3-mediated interaction that might prematurely terminate Itk signaling.

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REFERENCES

1. Kane, L. P., Lin, J., and Weiss, A. (2000) Curr. Opin. Immunol. 12, 242–249
2. Gibson, S., August, A., Kawakami, Y., Kawakami, T., Dupont, B., and Mills, G. B. (1996) J. Immunol. 156, 2716–2722
3. Heyeck, S. D., Wileyox, H. M., Bunnell, S. C., and Berg, L. J. (1997) J. Biol. Chem. 272, 25401–25408
4. Shan, X., and Wange, R. L. (1999) J. Biol. Chem. 274, 29223–29230
5. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998) Cell 92, 83–92
6. Liu, K.-Q., Bunnell, S. C., Gurniah, C. B., and Berg, L. J. (1998) J. Exp. Med. 187, 1721–1727
7. Miller, A. T., and Berg, L. J. (2000) J. Immunol. 164, 2163–2172
8. Takada, M., and Kurusaki, T. (1996) J. Exp. Med. 184, 31–40
9. Riggley, K. P., Harnett, M. M., Phillips, R. J., and Klaus, G. G. (1989) Eur. J. Immunol. 19, 2081–2086
10. Fluckiger, A. C., Li, Z., Kato, R. M., Wahl, M. I., Londero, G., Kinett, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998) EMBO J. 17, 1973–1985
11. Wang, D., Feng, J., Wen, J., Marine, J. C., Sangster, M. Y., Parnagas, E., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J., and Ile, J. (2000) Immunity 13, 25–35
12. Schaefer, E. M., and Schwartzberg, P. L. (2000) Curr. Opin. Immunol. 12, 262–268
13. Andreetti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997) Nature 385, 93–97
14. Braun, K. N., Fulton, D. B., and Andreetti, A. H. (2000) J. Biol. Chem. 275, 607–623
15. Nisitani, S., Sutterthwaite, A. B., Akashi, K., Weissman, I. L., Witte, O. N., and Wahl, M. I. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9274–9279
16. Park, H., Wahl, M. I., Afar, D. E., Turck, C. W., Rawlings, D. J., Tam, C., Scharenberg, A. M., Kinett, J. P., and Witte, O. N. (1998) Immunity 4, 515–525
17. Wahl, M. I., Fluckiger, A. C., Kato, R. M., Park, H., Witte, O. N., and Rawlings, D. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11526–11533
18. Kurasaki, T., and Kurusaki, M. (1997) J. Biol. Chem. 272, 15585–15598
19. Yang, W., and Desiderio, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 604–609
20. Hansson, H., Mattsson, P. T., Allard, P., Haapaniemi, P., Vihinen, M., Smith, C. I., and Hard, T. (1998) Biochemistry 37, 2912–2924
21. Hansson, H., Okoh, M. P., Smith, C. I., Vihinen, M., and Hard, T. (2001) FEBS Lett. 499, 67–70
22. Laederach, A., Cradid, K. W., Braun, K. N., Zamo, J., Fulton, D. B., Huang, W.-C., and Andreetti, A. H. (2000) Protein Sci. 11, 36–45
23. Bunnell, S. C., Diem, Y., Yaffe, M. B., Findell, P. R., Cantley, L. C., and Berg, L. J. (2000) J. Biol. Chem. 275, 2219–2230
24. Bunnell, S. C., Henry, P. A., Kalluri, R., Kirchhausen, T., Rickles, R. J., and Berg, L. J. (1996) J. Biol. Chem. 271, 25646–25656
25. Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J., and Ile, J. (1998) Immunity 13, 25–35
26. Hawley, R. G., Fong, A. Z., Burns, B. F., and Hawley, T. S. (1992) J. Exp. Med. 176, 1149–1163
27. Thomas, D. C., and Berg, L. J. (1997) J. Exp. Med. 185, 197–206
28. Nisitani, S., Kato, R. M., Rawlings, D. J., Witte, O. N., and Wahl, M. I. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2221–2226
29. Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A. C., Witte, O. N., and Kinett, J. P. (1998) Science 271, 823–825
30. August, A., Sadra, A., Dupont, B., and Hanafusa, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11227–11232
31. Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991) Cell 65, 435–441
32. Elhini, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, R. C., Ostergaard, H. L., and Stone, J. C. (2000)
Blood 95, 3199–3203
33. Zhang, W., Trible, R. P., Zhu, M., Liu, S. K., McGlade, C. J., and Samelson, L. E. (2000) J. Biol. Chem. 275, 23355–23361
34. Afar, D. E., Park, H., Howell, B. W., Rawlings, D. J., Cooper, J., and Witte, O. N. (1996) Mol. Cell. Biol. 16, 3465–3471
35. Pinschewer, D. D., Ochsenbein, A. F., Satterthwaite, A. B., Witte, O. N., Hengartner, H., and Zinkernagel, R. M. (1999) Eur. J. Immunol. 29, 2981–2987
36. Satterthwaite, A. B., Cheroutre, H., Khan, W. N., Sideras, P., and Witte, O. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13152–13157
37. Fowell, D. J., Shinkai, K., Liao, X. C., Beebe, A. M., Coffman, R. L., Littman, D. R., and Locksley, R. M. (1999) Immunity 11, 399–409
38. Shan, X., Czar, M. J., Bunnell, S. C., Liu, P., Liu, Y., Schwartzberg, P. L., and Wang, R. L. (2000) Mol. Cell. Biol. 20, 6945–6957
39. Liao, X. C., and Littman, D. R. (1995) Immunity 3, 757–769
40. Lucas, J. A., Atherly, L. O., and Berg, L. J. (2002) J. Immunol. 168, 6142–6151
41. Schaeffer, E. M., Broussard, C., Debnath, J., Anderson, S., McVicar, D. W., and Schwartzberg, P. L. (2000) J. Exp. Med. 192, 987–1000
42. Hao, S., and August, A. (2002) FEBS Lett. 525, 53–58
43. Larson, S. M., and Davidson, A. R. (2000) Protein Sci. 9, 2170–2180
44. Su, Y. W., Zhang, Y., Schweikert, J., Koretzky, G. A., Roth, M., and Wienands, J. (1999) Eur. J. Immunol. 29, 3702–3711
45. Zhang, W., Sommers, C. L., Burshtyn, D. N., Stubbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E., and Samelson, L. E. (1999) Immunity 10, 323–332
46. Ching, K. A., Grasis, J. A., Tailor, P., Kawakami, Y., Kawakami, T., and Tsukada, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6341–6346
47. Yamadori, T., Baba, Y., Matsushita, M., Hashimoto, S., Kurosaki, M., Kurosaki, T., Kishimoto, T., and Tsukada, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6341–6346
48. Tseng, S. R., Lou, Y. C., Pai, M. T., Jain, M. L., and Cheng, J. W. (2000) J. Biomol. NMR 16, 303–312