ZAP-70 Protein Tyrosine Kinase Is Constitutively Targeted to the T Cell Cortex Independently of its SH2 Domains

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Abstract. ZAP-70 is a nonreceptor protein tyrosine kinase that is essential for signaling via the T cell antigen receptor (TCR). ZAP-70 becomes phosphorylated and activated by LCK protein tyrosine kinase after interaction of its two NH_2-terminal SH2 domains with tyrosine-phosphorylated subunits of the activated TCR. In this study, the localization of ZAP-70 was investigated by immunofluorescence and confocal microscopy. ZAP-70 was found to be localized to the cell cortex in a diffuse band under the plasma membrane in unstimulated T cells, and this localization was not detectably altered by TCR stimulation. Analysis of mutants indicated that ZAP-70 targeting was independent of its SH2 domains but required its active kinase domain. The specific compartmentalization of ZAP-70 suggests that it may interact with an anchoring protein in the cell cortex via its hinge or kinase domains. It is likely that the maintenance of high concentrations of ZAP-70 at the cell cortex, that only has to move a short distance to interact with phophorylated TCR subunits, facilitates rapid initiation of signaling by the TCR. In addition, as the major increase in tyrosine phosphorylation induced by the TCR also occurs at the cell cortex (Ley, S.C., M. Marsh, C.R. Bebbington, K. Proudfoot, and P. Jordan. 1994. *J. Cell. Biol.* 125:639–649), ZAP-70 may be localized close to its downstream targets.

THE T cell antigen receptor (TCR)[1] is a complex oligomeric protein that may be considered as two functionally linked modules. The polymorphic disulfide-linked αβ heterodimer recognizes major histocompatibility complex molecules on the surface of antigen-presenting cells or target cells (Clevers et al., 1988). Noncovalently associated with the αβ heterodimer is the CD3 complex of polypeptides (γδε) and a disulfide-linked ζ homodimer. These associated polypeptides are responsible for the signal transduction function of the TCR (Weiss, 1993) and are also required for efficient surface expression of the intact receptor (Weiss, 1991). Stimulation of the TCR activates a cascade of biochemical events that triggers proliferation and differentiation of T cells into effector cells. The induced tyrosine phosphorylation of multiple intracellular proteins is one of the earliest detectable biochemical events after TCR stimulation (Klausner and Samelson, 1991). Pharmacologic and genetic experiments have established that the induction of protein tyrosine kinase (PTK) activity is essential for signaling via the TCR. However, the primary sequences of the identified components of the TCR contain no recognizable kinase domains. Rather, the TCR stimulates protein tyrosine phosphorylation by interacting sequentially with two different families of cytoplasmic nonreceptor PTKs (Weiss and Littman, 1994).

TCR signaling is initiated by receptor clustering, which results in the tyrosine phosphorylation of the cytoplasmic domains of the CD3 complex subunits (Qian et al., 1993; Sancho et al., 1993; Straus and Weiss, 1993) and ζ homodimers in specific motifs (Baniyash et al., 1988), termed immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAM sequence is defined as two tyrosine residues spaced 9 to 11 residues apart with isoleucine or leucine residues positioned three residues COOH terminal to each tyrosine (YXXL X_{6-8} YXXL; Weiss, 1993). Each of the CD3 subunits contains one ITAM, whereas the ζ chain contains three tandemly arranged ITAMs. Studies in transfected COS cells (Iwashima et al., 1994) and in mutant T cell lines have suggested that ITAM phosphorylation is mediated by LCK, a member of the SRC family of cytoplasmic PTKs (Karnitz et al., 1992; Straus and Weiss, 1992). Consistent with this hypothesis, both constitutive and inducible tyrosine phosphorylation of ζ is abolished in LCK null thymocytes (van Oers et al., 1996*a*). Targeted disruption

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1. *Abbreviations used in this paper*: ITAM, immunoreceptor tyrosine-based activation motifs; PTK, protein tyrosine kinase; TCR, T cell antigen receptor.

of the *lck* gene also results in a substantial reduction in the development of $CD4^+CD8^+$ thymocytes, demonstrating its importance in T cell development (Molina et al., 1992). Gene knockout experiments in mice have also indicated that FYN is functionally important in TCR signaling in mature thymocytes, although it is not absolutely required for αβ T cell development (Appleby et al., 1992; Stein et al., 1992). However, combined disruption of *lck* and *fyn* genes completely arrests αβ T cell development at the $CD4^-CD8^-$ stage, suggesting that the function of LCK and FYN in T cell development is only partially redundant (Groves et al., 1996; van Oers et al., 1996*b*). Thus FYN may also contribute to TCR ITAM phosphorylation in certain circumstances.

Tyrosine phosphorylation of ITAMs of the CD3 complex and the ζ homodimers recruits a second family of cytoplasmic PTKs to the TCR, comprising ZAP-70 and SYK (Taniguchi et al., 1991; Chan et al., 1994*b*). The association of these PTKs with the TCR is mediated by a high affinity interaction between their two NH_2-terminal SH2 domains with the two phosphorylated tyrosines in an ITAM (Chan et al., 1992, 1994*a*; Straus and Weiss, 1993; Wange et al., 1993). This interaction requires both of the SH2 domains (Bu et al., 1995; Isakov et al., 1995). After binding to the TCR, ZAP-70 and SYK themselves become tyrosine phosphorylated and are then activated. For ZAP-70, this phosphorylation is thought to be primarily mediated by LCK (Chan et al., 1995; Wange et al., 1995), whereas the binding of SYK to ITAMs itself has been shown to stimulate autophosphorylation and subsequent activation (Rowley et al., 1995*a*; Shiue et al., 1995). In thymocytes, ZAP-70 is constitutively associated with the ζ chain but is not tyrosine phosphorylated and activated (van Oers et al., 1994). In these cells, the triggering event in TCR signaling does not appear to be the binding of ZAP-70 to the ζ chain. Rather, it is the colocalization of CD4–LCK complexes with the TCR after stimulation which then facilitates the phosphorylation of ζ-associated ZAP-70 by LCK (Wiest et al., 1993, 1996).

Once activated, ZAP-70 becomes autophosphorylated on multiple tyrosines (Watts et al., 1994; Chan et al., 1995). This generates binding sites that recruit SH2-containing signaling proteins to the plasma membrane, which include LCK (Duplay et al., 1994), Vav (Katzav et al., 1994; Huby et al., 1995), Ras-GAP, abl (Neumeister et al., 1995), and a FAK-related PTK (Kanner et al., 1994), where they can become tyrosine phosphorylated or brought into proximity with their substrates. Subsequently, downstream effector functions are triggered, including the activation of Ras and the mobilization of intracellular Ca^{2+} (Weiss and Littman, 1994). Genetic studies in both murine and human systems have confirmed the importance of ZAP-70 in TCR signaling and T cell development (Hivroz and Fischer, 1994; Negishi et al., 1995). In contrast, SYK does not appear to be essential for αβ T cell development (Turner et al., 1995), although the development of epithelial γδ T cells is disrupted in SYK-negative mice (Mallick-Wood et al., 1996). Recent experiments have also indicated that SYK does not require either LCK or CD45 expression to function in TCR signaling, unlike ZAP-70 (Chu et al., 1996). Thus the activation requirements for SYK are different from ZAP-70, suggesting a distinct role in TCR signaling.

Earlier experiments from one of our laboratories demonstrated that LCK is predominantly targeted to the plasma membrane in mature human T cells (Ley et al., 1994*a*). In contrast, FYN was found to be localized adjacent to the microtubule cytoskeleton and was not detected at the plasma membrane unless overexpressed by transfection, raising the possibility that it might carry out functions that are distinct from LCK. The major increase in tyrosine phosphorylation that occurs after TCR stimulation was found to take place in the T cell cortex coincident with LCK localization. This suggested that LCK might contribute to the increase in cortical tyrosine phosphorylation, consistent with its proposed role in phosphorylating TCR ITAMs after stimulation. In this study, the intracellular localization of ZAP-70 was investigated by immunofluorescence and confocal microscopy. These experiments indicated that ZAP-70 was also targeted to the T cell cortex, in a constitutive fashion, independently of its SH2 domains. The implication of these results for the function of ZAP-70 is discussed.

Materials and Methods

Cell Culture and Transfections

The E6.1 Jurkat T cell line was cultured in RPMI medium supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a rapid growth phase before staining or transfection. Transient transfection was carried out by electroporation as described previously (Salmeron et al., 1996). Briefly, cells were washed three times in serum-free RPMI medium and were then resuspended at 1×10^8 cells/ml. 250 μl was transferred into a gene pulser cuvette (BioRad Laboratories, Hertfordshire, UK) and 10 μg of the appropriate plasmid was added. Cells were pulsed at 960 μF and 250 V and left for 10 min at room temperature (RT). Cells were then transferred into 10 ml of RPMI medium supplemented with 10% FCS and cultured for 20 h before harvesting.

3T3 fibroblasts were maintained in Dulbecco's minimal essential medium, supplemented with 10% newborn calf serum, and the above concentrations of L-glutamine and antibiotics. Cells were split 1/4–1/5 every 3 d and were not allowed to reach confluency. Before a transfection, cells were plated at 1.25×10^5 cells/well of a 6-well plate (Costar Corp., Cambridge, MA) and cultured overnight. Cells were transfected using Lipofectamine (GIBCO BRL, Gaithersburg, MD) following the manufacturer's instructions. 0.5 μg of DNA was mixed with 0.5 μl of Lipofectamine per transfection, and cells were incubated for 6 h with this complex before washing and transferring into complete medium. Cells were then cultured for an additional 18 h before harvesting and staining.

DNA Constructs

The generation of the NH_2-terminal hemagglutinin (HA) epitope-tagged ZAP-70, the COOH-terminal HA epitope-tagged ZAP-70 construct, the kinase-inactive K369A mutant, and the phosphorylation site mutants (Y292F, Y492F, Y493F, and YY492,493FF) has been described previously (Chan et al., 1992, 1995; Iwashima et al., 1994; Wange et al., 1995). In the ΔSH2–ZAP-70 mutant both of the SH2 domains were deleted. To do this the SacII–StuI DNA fragment encoding the SH2 domains (residues 220–964 of the original cDNA) was replaced with the following oligonucleotides: (+)5′-GGCGCACCTGCCCTTCTACCCATACGATGTTCCAGATTACGCTG-3′ and (-)5′-CGCCGCGTGGACGGGAAGATGGGTATGCTACAAGGTCTAATGCGAC-3′. The resulting construct encoded the first five amino acids of ZAP-70 followed by nine amino acids from the HA epitope and then the remaining COOH-terminal part of ZAP-70. The ΔSph–ZAP-70 construct, in which the majority of the kinase domain was deleted (residues 1288–1988 of the cDNA), was generated by excising the Sph1 fragment from the NH_2-terminally HA-tagged ZAP-70 construct and religating. All of the ZAP-70 constructs were subcloned into the $pcDNA3^{neo}$ vector for transient transfection experiments.

HA epitope-tagged SYK was also subcloned into the pcDNA3^{neo} vector for transient transfection experiments (Rowley et al., 1995*b*).

Antibodies

The ZAP-4 antiserum, which was raised against a synthetic peptide corresponding to residues 271–290 of human ZAP-70 (Huby et al., 1995), was used for staining and immunoprecipitation of endogenous ZAP-70. The 12CA5 mAb (Hsi et al., 1989) was used for immunoprecipitation, immunoblotting (1 μg/ml), and immunohistological staining (0.3 μg/ml) of HA epitope-tagged ZAP-70. The LCK-1 anti-peptide antibody was used for immunofluorescent staining of LCK at a dilution of 1:1000 in PBS (Koegl et al., 1994). Tyrosine phosphorylated proteins were detected using the 4G10 anti-PTyr mAb at 1 μg/ml for both immunofluorescence and Western blotting (from B. Druker, Oregon Health Sciences University, Portland, OR). The rat anti–α-tubulin mAb, YOL34, was used at 1:20 dilution and was obtained from Serotec (Oxford, UK). Polymerized actin was detected using rhodamine-coupled phalloidin at 1 μg/ml (Sigma Chemical Co., Dorset, UK).

The fluorochrome-labeled antibodies used for immunofluorescent staining of cells were all obtained from Jackson Immunoresearch Labs (West Grove, PA). Goat anti–rabbit Ig coupled to FITC was used for labeling with ZAP-4 antibody. For labeling with 12CA5 mAb, goat anti–mouse Ig coupled to either fluorescein or Texas red was used as a second-stage antibody. Goat anti–rat Ig coupled to Texas red was used for α-tubulin staining with the YOL34 mAb.

Immunofluorescence and Confocal Microscopy

To stain for endogenous ZAP-70, cells were first settled onto coverslips, which had been pretreated with 3-aminopropyltriethoxy silane (TESPA; Sigma Chemical Co.), for 30 min at RT and fixed in 2% paraformaldehyde in PBS for 6 min. In experiments in which cells were double labeled for PTyr, 1 mM Na_3VO_4 was added to the fixing buffer. Cells were then incubated with 0.5% BSA–PBS for 1 h and permeabilized with 0.2% saponin in PBS for 1 h. Permeabilized cells were incubated with ZAP-4 antiserum (1:1000 dilution) overnight and then washed three times in PBS. The cells were then incubated with anti–rabbit Ig–FITC antibody for 1 h, washed extensively in PBS, and mounted onto glass slides using a glycerol/PBS solution (Citifluor; Agar Scientific, UK).

For immunofluorescent staining of HA–ZAP-70, transiently transfected Jurkat T cells were washed with PBS and then fixed for 1 h in 3.7% paraformaldehyde in PBS at RT. Fixed cells were then settled onto TESPA-treated coverslips. Transfected 3T3 cells were directly cultured on coverslips placed in the culture wells. Cells were fixed in 3.7% paraformaldehyde in PBS for 1 h at RT. Coverslips were then incubated with 0.1% Triton X-100 in PBS for 5 min to permeabilize the attached cells and blocked for 15 min with 0.5% fish skin gelatin. After blocking, the coverslips were washed in PBS and then incubated for 1–2 h with primary antibody at the appropriate concentration, followed by FITC or Texas red-conjugated second-stage antibody for 1 h. Nonspecifically bound antibody was removed by extensive washing in PBS, and the coverslips were then mounted onto glass slides as above.

Confocal imaging was performed using a laser scanning head (MRC-600; BioRad Laboratories) fitted to an Olympus BH-2 microscope. Samples were excited at 488 and 568 nm and fluorescein and Texas red signals detected through the K2 and K1 filter blocks, respectively. Images were collected using the photon counting mode of the COMOS program (Bio-Rad Laboratories). In double labeling experiments, bleedthrough of the Texas red signal into the fluorescein channel was negligible. To visualize cell nuclei, permeabilized cells were stained with 7–amino-actinomycin D (Molecular Probes, Eugene, OR) at 50 μg/ml together with the primary antibody. The stained cells were then excited at 568 nm, and images were collected through the K1 filter. Separate images of fluorescein-stained antigens were then collected by exciting at 488 nm and a collection of images through the K2 filter.

Cell Fractionation

To separate soluble and particulate cell fractions, 1×10^7 Jurkat T cells were resuspended in 1 ml of hypotonic buffer (10 mM Tris, 2 mM EDTA, and 1 mg/ml each of chymostatin, leupeptin, and pepstatin, pH 7.4) and then subjected to two successive freeze/thaw cycles. Cells were then disrupted by homogenization on ice using a tight fitting Dounce homogenizer (40 strokes), and the salt concentration was then adjusted to 150 mM NaCl. Intact cells, nuclei, and cytoskeleton were pelleted by two successive centrifugations at 480 *g* for 5 min. This pellet, termed P1, was washed in hypotonic buffer and then resuspended in Laemmli sample buffer. The supernatant from the low speed centrifugation was recentrifuged at 100,000 *g* for 30 min to generate a second pellet fraction, termed P2, which was enriched for membranes. The supernatant from this high speed centrifugation, termed S2, contained soluble proteins. Equivalent portions of the fractionated protein were resolved by SDS-PAGE and Western blotted with anti–ZAP-70 antiserum.

To determine the fraction of ZAP-70 that remained associated with cells after paraformaldehyde fixation and permeabilization, 1×10^7 Jurkat T cells were incubated for 1 h in 3.7% paraformaldehyde in PBS. Control cells were incubated in PBS alone. The cells were then pelleted, permeabilized by addition of 1 ml of 0.1% Triton X-100 in PBS, and incubated for 5 min at RT. Insoluble material was pelleted by centrifugation and the supernatant aspirated (S fraction) and mixed 1:1 with reducing sample buffer. The pellet was washed once in the lysis buffer and then solubilized in 200 μl of reducing sample buffer (P fraction). Equivalent amounts of the S and P fractions were then resolved by SDS-PAGE and Western blotted with anti–ZAP-70 antibody.

Immunoprecipitation and Western Blotting Analysis

Transfected Jurkat T cells were washed in serum-free RPMI and then stimulated with OKT3 CD3 mAb for 5 min at 37°C. After pelleting by centrifugation, cells were lysed with 1 ml of ice-cold immunoprecipitation buffer (IPB; 150 mM NaCl, 20 mM Tris-HCl, 1% NP-40, 100 mM Na_3VO_4, 5 mM NaF, and 5 μg/ml each of chymostatin, leupeptin, and pepstatin, pH 7.4) for 15 min at 4°C. Cell lysates were cleared of insoluble debris by centrifugation at 13,000 *g* for 15 min at 4°C and then precleared once by incubation with 10 μl of protein-A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) for 15 min at 4°C. For immunoprecipitation of HA–ZAP-70, 10 μg of 12CA5 mAb were coupled to 10 ml of protein-A Sepharose with dimethylpimelimidate (Schneider et al., 1982) and incubated with precleared cell lysate for 4 h or overnight. Endogenous ZAP-70 was immunoprecipitated using ZAP-4 antiserum as described previously (Huby et al., 1995). After six washes with ice-cold IPB, immunoprecipitated protein was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Western blotting was carried out as described previously (Ley et al., 1994*b*). PVDF membranes were stripped of bound antibody using the ECL protocol (Amersham, Buckinghamshire, UK) in experiments in which blots were probed for multiple antigens.

Results

ZAP-70 Is Constitutively Targeted to the T Cell Cortex in Jurkat T Cells

In initial experiments, the localization of endogenous ZAP-70 was investigated using a polyclonal anti–ZAP-70 peptide antiserum, ZAP-4, to stain the endogenous protein in Jurkat T cells. In Fig. 1 *A*, it can be seen that staining was detected at the cell cortex and also in the cytoplasm. Several other anti–ZAP-70 antisera and two anti–ZAP-70 mAb were also tested for immunofluorescence. However, none of these produced any specific staining. The addition of specific immunizing ZAP-4 peptide during incubation with the primary antibody reduced staining at the cell cortex to background levels, and staining was also markedly reduced in the cytoplasm (Fig. 1 *B*). The cytoplasmic staining was also detected in a B cell line, which does not contain ZAP-70, suggesting that this resulted from cross-reaction with an irrelevant antigen (data not shown). Taken together, these data suggested ZAP-70 was localized to the cell cortex in unstimulated Jurkat T cells.

Since endogenous ZAP-70 was difficult to detect by immunofluorescence, it was important to confirm its cortical localization. To do this, Jurkat T cells were transiently transfected with a ZAP-70 cDNA in which an HA epitope

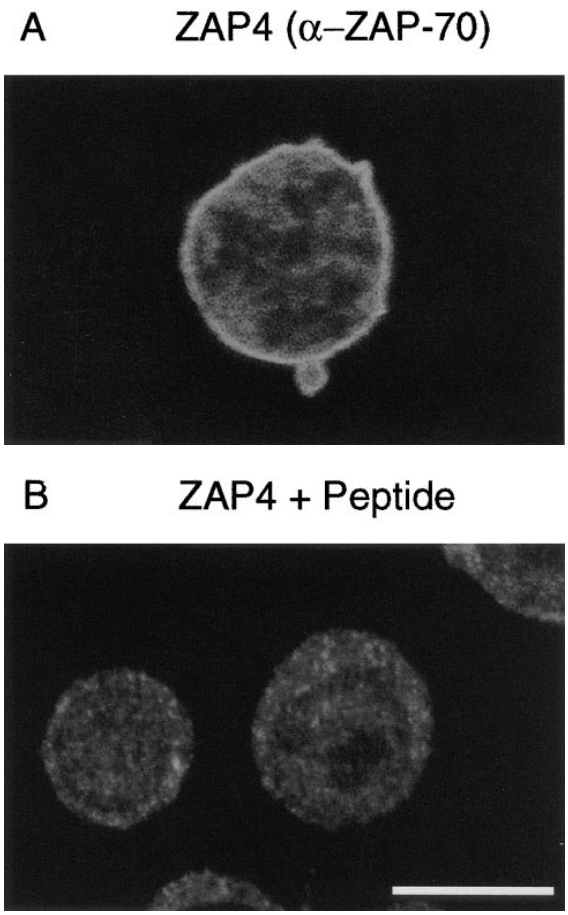


Figure 1. Localization of endogenous ZAP-70 in Jurkat T cells. Staining with ZAP 4 anti–ZAP-70 antiserum in Jurkat T cells reveals specific cortical staining (*A*) which was abrogated by the addition of blocking peptide, to which the antiserum was raised during the primary staining step (*B*). Low levels of nonspecific staining were also detected in the cytoplasm. Bar, 10 μm.

tag had been spliced onto its COOH terminus and then stained using an anti-HA mAb. In Fig. 2 *A*, it can be seen that transiently transfected ZAP-70–HA was concentrated at the cell cortex in a diffuse band. Counterstaining with 7–amino-actinomycin D, to reveal the position of the nucleus, clearly indicated that between the cell cortex and the cell nucleus there was cytoplasm that contained only low levels of ZAP-70–HA (Fig. 2 *A*). Similar results were obtained using methanol/acetone to fix the Jurkat cells (data not shown), indicating that the cortical localization of ZAP-70–HA was not an artifact of the paraformaldehyde-fixation procedure. Staining for transfected LCK produced very sharp staining at the cell cortex in contrast to transfected ZAP-70–HA (Fig. 2 *C*). This difference in staining was also evident by measuring the pixel intensity along a transect through a cell stained for ZAP-70–HA or LCK (Fig. 2, *B* and *D*, respectively). The LCK staining profile produced a narrow peak that was symmetrical. In contrast, the peak for ZAP-70–HA staining was broader than for LCK and asymmetrically distributed, with highest intensity towards the plasma membrane and reduced staining moving into the cell. The asymmetric distribution of ZAP-70–HA suggested that the breadth of the ZAP-70–HA staining was not simply an artifact of its brightness. By setting the background fluorescence levels against an untransfected cell, it was also evident that ZAP-70–HA staining was detected at low levels throughout the cytoplasm. However, the brightest staining was all concentrated at the cell cortex.

Endogenous ZAP-70 localized in a tight band at the cortex, in contrast to the broad staining pattern detected with transfected ZAP-70–HA. However, the staining achieved with the transfected ZAP-70–HA (Fig. 2 *A*) was considerably brighter than that with the endogenous protein (Fig. 1 *A*). By artificially decreasing the intensity of staining for the transfected protein using the COMOS software, so that only the highest levels of ZAP-70–HA were detected, the staining pattern for transfected ZAP-70–HA became very similar to the endogenous protein (data not shown), suggesting that the difference was quantitative rather than qualitative. In addition, untagged ZAP-70 transfected into Jurkat T cells had the same distribution as ZAP-70–HA, revealed using the ZAP-4 antiserum (data not shown), indicating that the addition of an HA tag did not affect ZAP-70 localization. Both endogenous ZAP-70 and transfected HA-ZAP–70 associated with phospho-ζ and were tyrosine phosphorylated after stimulation of the cells with CD3 antibody (Fig. 2 *E*). Taken together, these data indicate that transfected ZAP-70–HA behaved in a similar fashion to the endogenous protein in terms of its localization and also its inducible interaction with the TCR.

The specific localization of ZAP-70 to the cell cortex suggested that it might be associated either with the plasma membrane or cortical cytoskeleton. To determine the solubility of ZAP-70, Jurkat T cells were lysed in hypotonic buffer by Dounce homogenization and then separated into P1 (nuclei and cytoskeleton), P2 (membrane), and S2 (soluble) fractions (as described in Materials and Methods). Western blotting of these fractions demonstrated that virtually all ZAP-70 was soluble, and only a small fraction was detected in the two particulate fractions (Fig. 3 *A*). No change in the fractionation of ZAP-70 was detected after TCR stimulation for 5 or 20 min. These data raised the possibility that paraformaldehyde fixation might not have rendered the majority of ZAP-70 insoluble, such that it was lost from the cell after Triton X-100 extraction. In this case, immunofluorescent staining would only have detected the small percentage that was already associated with the particulate fraction. Alternatively, it was possible that the putative interaction of ZAP-70 with the particulate fraction was not stable after cell lysis. To investigate whether immunofluorescent staining detected all ZAP-70 after fixation, Jurkat T cells were fixed with 3% paraformaldehyde or left unfixed and were then extracted with 0.1% Triton X-100. Western blotting of soluble and insoluble protein confirmed that paraformaldehyde treatment rendered essentially all ZAP-70 insoluble, confirming that the cortical ZAP-70 staining corresponded to essentially all of ZAP-70 expressed in Jurkat T cells (Fig. 3 *B*). These data were, therefore, consistent with the hypothesis that interaction of ZAP-70 with the cell cortex was not stable after cell lysis.

In summary, the data in this section indicated that ZAP-70 was not uniformly distributed throughout the cytoplasm

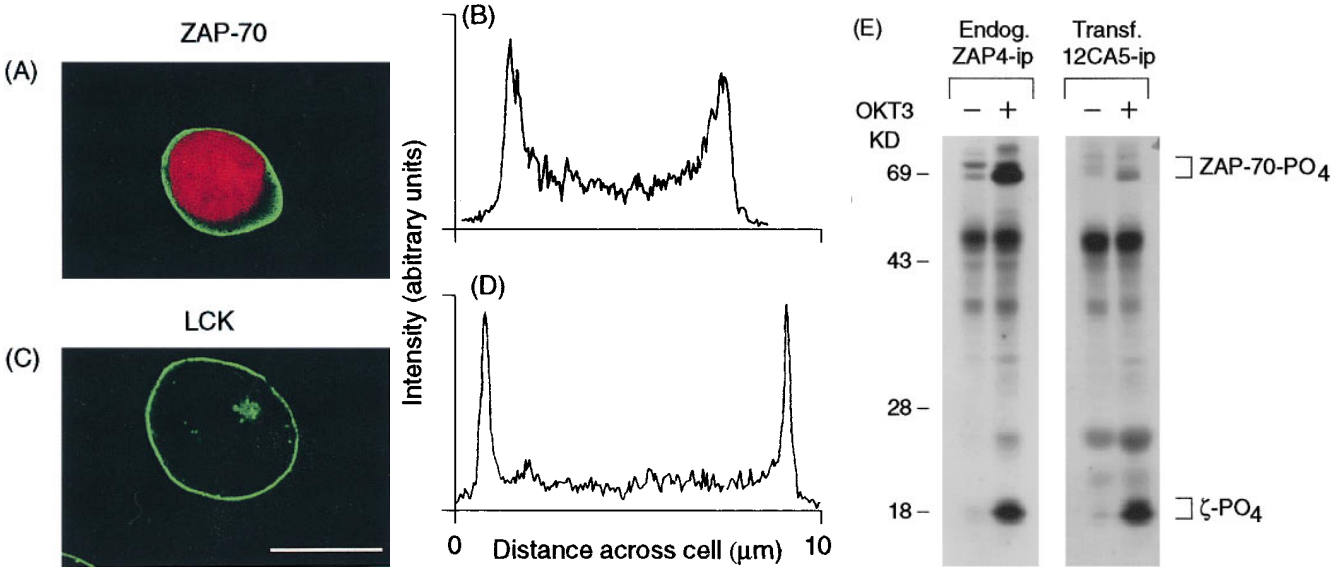


Figure 2. Localization of HA-tagged ZAP-70 after transient transfection into Jurkat T cells. Transfected cells were double stained with anti-HA tag mAb 12CA5 (*green*) and the nuclear stain 7–amino-actinomycin D (*red*), revealing that ZAP-70–HA expression is limited primarily to the cell cortex, with maximal staining proximal to the plasma membrane (*A*). A gradient of ZAP-70–HA staining intensity measured according to pixel brightness was quantified along a cell transect as shown in (*B*). In comparison, very bright LCK staining is seen after transient transfection with an LCK expression plasmid, which nevertheless remains tightly and symmetrically associated with the plasma membrane (*C*), illustrated quantitatively by the corresponding cell transect (*D*). Transfected ZAP-70–HA is shown to behave similarly to endogenous ZAP-70 after TCR activation, becoming tyrosine phosphorylated and associated with phosphorylated TCR ζ (*E*). Bar, 10 μm.

but rather was specifically targeted to the cell cortex in a diffuse band under the plasma membrane in unstimulated T cells.

Stimulation of the TCR Does Not Alter the Gross Localization of ZAP-70

As outlined in the introduction, TCR stimulation induces phosphorylation of ζ and CD3 ITAMs which creates docking sites for ZAP-70. To investigate whether the cortical localization of ZAP-70 was altered after TCR stimulation, Jurkat T cells were stimulated with CD3 mAb or left unstimulated and then fixed with paraformaldehyde. The cells were then double labeled for endogenous ZAP-70 and PTyr. Stimulation of the TCR with CD3 antibody did not alter the localization of ZAP-70 over a period of 5 min, although there was a clear transient induction of PTyr proteins at the cell cortex (Fig. 4, *A* and *B*), and as reported previously (Ley et al., 1994*a*). The localization of transfected HA–ZAP-70 after CD3 mAb stimulation was also unchanged after stimulation with CD3 antibody (data not shown).

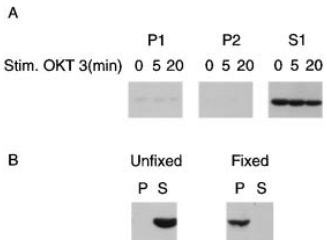


Figure 3. ZAP-70 is largely soluble before fixation. Jurkat T cells were disrupted by Dounce homogenization (*A*) and separated into three fractions; (*P1*) pellet recovered by 280 *g* centrifugation, comprised primarily of nuclear debris and the actin cytoskeleton; (*P2*) pellet recovered at 100,000 *g* comprised membranous fragments, protein aggregates, and the remaining soluble fraction S1. ZAP-70 compartmentalization was unaffected by stimulation of cells for 5 or 20 min with OKT3 $F(ab)_2$ before homogenization. After fixation for 5 min in 2% paraformaldehyde, essentially all ZAP-70 became insoluble (*B*) and was recovered in the pellet P rather than the soluble fraction S after centrifugation for 2 min at 8,000 *g*.

Localization of ZAP-70 Is Distinct from Cortical Actin and Microtubule Cytoskeletons

The targeting of ZAP-70 to the cell cortex raised the possibility that it might be associated with the cortical actin cytoskeleton. However, double labeling for HA–ZAP-70 and F-actin, using rhodamine-coupled phalloidin, demonstrated that the patterns of staining were distinct (Fig. 5 *A*). Thus, similar to the results with LCK, phalloidin staining produced relatively sharp staining, in contrast to HA–ZAP-70 staining which was more diffuse. This difference was also evident when pixel intensity was plotted along a transect through a cell double labeled for ZAP-70–HA and actin (Fig. 5 *B*). These data suggested that the cortical localization of ZAP-70 was not mediated via its direct interaction with the cortical actin cytoskeleton.

Recent data from one of our laboratories has indicated that a fraction of ZAP-70 is associated with αβ tubulin in cell lysates (Huby et al., 1995). However, double labeling for ZAP-70–HA and α-tubulin demonstrated that ZAP-70 localization was clearly distinct from that of microtubules, which were detected throughout the cytoplasm and concentrated adjacent to the nucleus at the centrosome (Fig.

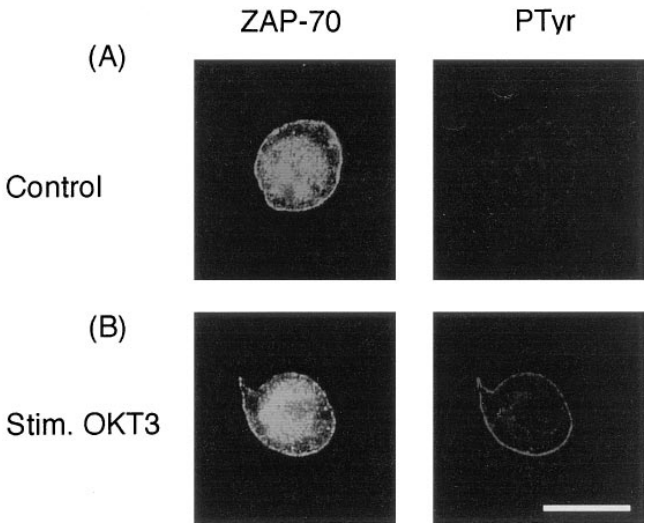
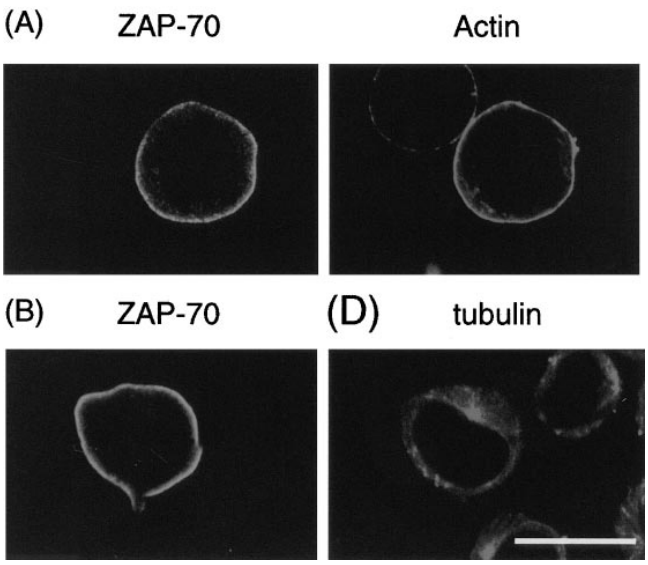


Figure 4. Localization of ZAP-70 is unaffected by TCR stimulation. Jurkat T cells were either unstimulated (*A*), or stimulated for 5 min with $F(ab)_2$ fragments of the CD3 mAb, OKT3 (*B*), fixed, and double stained for endogenous ZAP-70 (*left*) and total phosphotyrosine (*right*). Staining for endogenous ZAP-70 with ZAP-4 antiserum also produced cytoplasmic fluorescence which was nonspecific (Fig. 1). Bar, 10 μm.



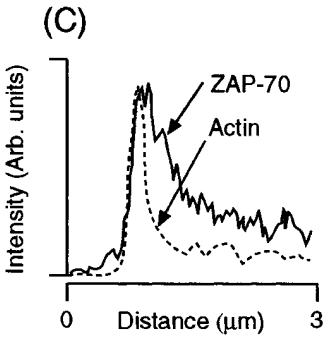


Figure 5. ZAP-70 does not colocalize with actin or α-tubulin. ZAP-70–HA transfected Jurkat T cells were double stained to reveal ZAP–70 and actin microfilaments in the same cell (*A*). ZAP-70–HA gave a broader staining pattern, quantified by the pixel density transect shown in *B*. Double staining was similarly used to reveal ZAP-70–HA and α-tubulin distribution within the same cells (*C*). Bar, 10 μm.

5 *C*). These data suggested that ZAP-70 was not targeted to the cell cortex via an association with the microtubule cytoskeleton.

Cortical Localization of ZAP-70 in Transfected 3T3 Fibroblasts

To investigate whether the cortical targeting of transfected ZAP-70–HA was specific to T cells, 3T3 fibroblasts were transiently transfected with a ZAP-70–HA plasmid vector. ZAP-70–HA was detected exclusively in the cell cortex in these cells (Fig. 6 *A*). Double labeling with phalloidin revealed that ZAP-70–HA had a similar distribution to the cortical actin, albeit more diffuse, as in Jurkat T cells (Fig. 6 *B*). No ZAP-70–HA staining was detected on actin stress fibers or on microtubules (data not shown). Disruption of actin filaments with cytochalasin D did not delocalize ZAP-70–HA from the cell cortex, although cell morphology was dramatically altered (Fig. 6, *C* and *D*). Cytochalasin D also had no effect on ZAP-70 localization in Jurkat T cells (data not shown). Taken together, these data indicated that targeting of ZAP-70 to the cell cortex did not require any other lymphoid-specific proteins and was not determined via a direct interaction with cortical actin.

ZAP-70 Is Targeted to the Cell Cortex Independently of its SH2 Domains

Since ZAP-70 interacts with ITAMs of the TCR via its two SH2 domains after CD3 mAb stimulation (Weiss and Littman, 1994), the localization of ZAP-70 to the cell cortex in unstimulated Jurkat T cells suggested that this targeting was not due to SH2 binding to tyrosine-phosphorylated TCR. The cortical localization of ZAP-70 in 3T3 fibroblasts, which do not express TCRs, supported this hypothesis (Fig. 6). However, it was still possible that ZAP-70 was anchored to the cortical cytoskeleton via the binding of its SH2 domains to other tyrosine-phosphorylated proteins. To investigate this possibility, the localization was tested of a COOH terminally HA-tagged mutant of ZAP-70, ΔSH2-ZAP-70–HA, in which both SH2 domains had been deleted. This mutant localized in a diffuse band at the cell cortex in both Jurkat T and 3T3 cells (Fig. 7, *B* and *F*, respectively) similarly to wild type ZAP-70–HA (Fig. 7, *A* and *E*).

To investigate whether the kinase domain of ZAP-70 was involved in intracellular targeting, the localization of the ΔSph-ZAP-70–HA mutant was determined, in which the majority of the kinase domain (residues 358 to 593) was deleted. ΔSph-ZAP-70–HA was detected throughout the cytoplasm in both Jurkat T cells and 3T3 cells, suggesting that the kinase domain played a role in ZAP-70 localization (Fig. 7, *C* and *G*). To investigate whether the kinase activity of ZAP-70 was important in its intracellular targeting, the localization of the kinase-inactive K369A-ZAP-70–HA mutant was determined. K369A-ZAP-70–HA had a similar localization to the ΔSph-ZAP-70–HA mutant (Fig. 7, *D* and *H*). Taken together, the data in this section suggested that cortical localization of ZAP-70 required its active kinase domain but was independent of its SH2 domains.

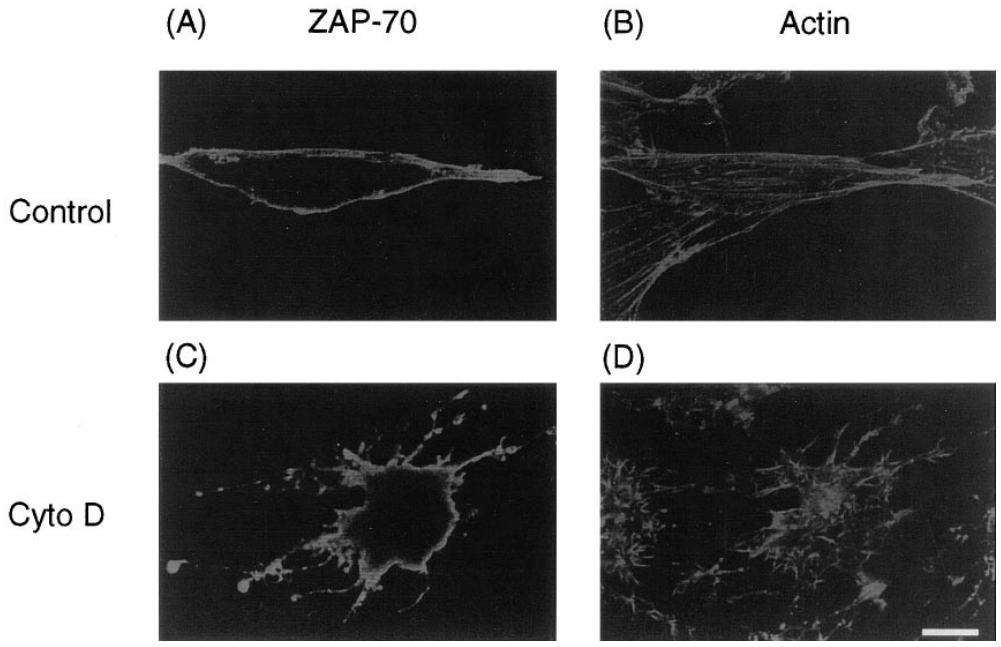


Figure 6. ZAP-70 localization is unaffected by disruption of the actin cytoskeleton. NIH 3T3 fibroblasts were transfected with a plasmid encoding ZAP-70–HA, fixed, and double stained to reveal the distribution of ZAP-70–HA (*A*) and actin microfilaments (*B*) in the same cells. Treatment of cells with cytochalasin D before fixation did not delocalize ZAP-70–HA from the cell cortex (*C*), although the actin cytoskeleton was disrupted (*D*). Bar, 10 μm.

Mutation of the Major Inducible Sites of Phosphorylation of ZAP-70 Does Not Affect its Cortical Targeting

The requirement for an active kinase domain for ZAP-70 to localize to the cell cortex raised the possibility that autophosphorylation by ZAP-70 might be important for its targeting. This could potentially create binding sites for putative SH2-containing proteins in the cell cortex with which it could interact and be targeted. After TCR stimulation, ZAP-70 is known to be inducibly phosphorylated on three tyrosine residues: Y292, Y492, and Y493 (Watts et al., 1994; Chan et al., 1995; Wange et al., 1995). Analysis of point mutants has indicated that phosphorylation of Y493 is required for the induction of ZAP-70 tyrosine kinase activity by the TCR (Chan et al., 1995; Wange et al., 1995; Kong et al., 1996). In contrast, tyrosine phosphorylation of Y292 and Y492 appears to negatively regulate the activity of ZAP-70 (Wange et al., 1995; Kong et al., 1996; Zhao and Weiss, 1996). Current data suggest that phosphorylation of Y493 is mediated by LCK, whereas phosphorylation of Y292 and Y492 is the consequence of autophosphorylation (Wange et al., 1995; Kong et al., 1996).

As an initial step to investigate the possible role of autophosphorylation of ZAP-70 in its localization, the distribution of several point mutants of HA–ZAP-70 was determined, in which the known phosphorylated tyrosines described above were individually substituted for phenylalanine. In Fig. 8, it can be seen that all four of the point mutants localized to the cell cortex both in transiently transfected Jurkat T cells and 3T3 fibroblasts, similar to the wild-type HA–ZAP-70. These data, therefore, indicated that phosphorylation of tyrosines 292, 492, and 493 on ZAP-70 was not required for its cortical targeting.

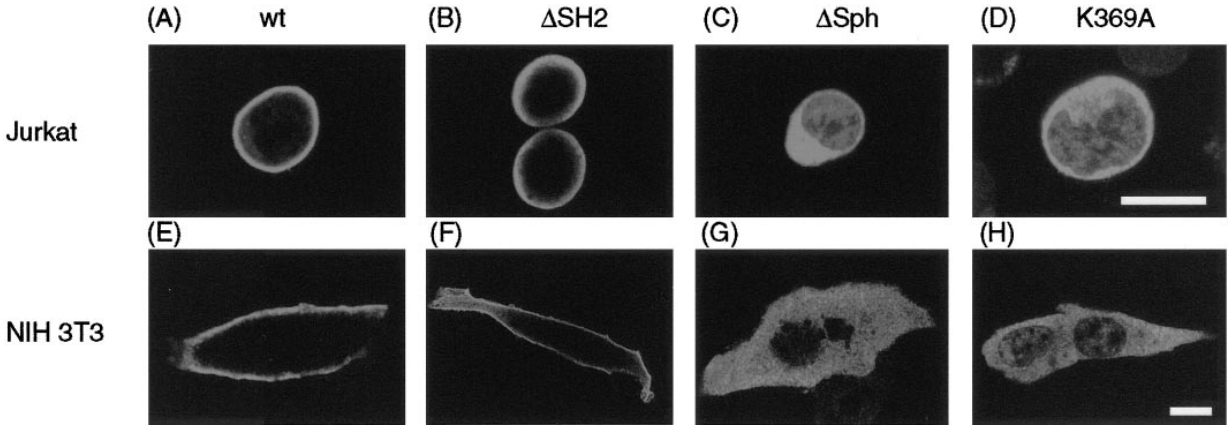


Figure 7. ZAP-70 requires an active kinase domain for its localization. Jurkat T cells (*A–D*) or NIH 3T3 fibroblasts (*E–H*) were transfected with plasmids encoding either: wild-type ZAP-70–HA (*A* and *E*), ΔSH2-ZAP-70–HA (*B* and *F*), ΔSph-ZAP-70–HA (*C* and *G*), or kinase-dead ZAP-70.K369A–HA. Transfected cells were stained with 12CA5 to reveal the HA-tagged ZAP-70 protein. Bars, 10 μm.

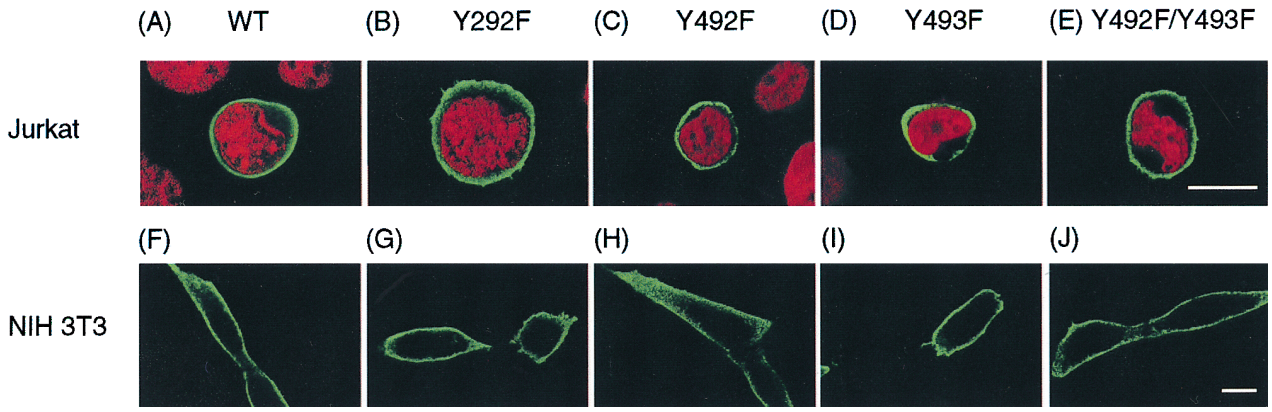


Figure 8. Inducibly phosphorylated tyrosine residues are not involved in ZAP-70 localization to the cortex. Jurkat T cells (*A–E*) or NIH 3T3 fibroblasts (*F–J*) were transfected with plasmids encoding either wild-type ZAP-70 (*A* and *F*), Y292F (*B* and *G*), Y492F (*C* and *H*), Y493F (*D* and *I*), or Y492F/Y493F (*E* and *H*). Unlike previous experiments, all constructs were HA-tagged at the NH_2 terminus. Transfected cells were stained with 12CA5 mAb to reveal HA-tagged ZAP-70 protein (*green*) and in the case of Jurkat were counterstained with 7–amino-actinomycin D to reveal the position of the nucleus (*red*). Bars, 10 μm.

SYK Is Constitutively Targeted to the Cell Cortex in Jurkat T Cells and 3T3 Fibroblasts

The protein tyrosine kinase SYK is 60% identical to ZAP-70 in humans (Law et al., 1994). Similar to ZAP-70, SYK can associate with phospho-ζ in murine thymocytes and in Jurkat T cells (Chan et al., 1994*a*; Thome et al., 1995; van Oers et al., 1995). Furthermore, ZAP-70 can complement SYK function in a SYK-negative B cell line (Kong et al., 1995). The similarities of SYK to ZAP-70 raised the possibility that it might also be localized constitutively to the cell cortex. E6.1 Jurkat T cells do not express SYK (Fargnoli et al., 1995). Therefore, HA epitope-tagged SYK was transiently expressed by transfection in this cell line. HA-SYK was detected in a band at the cell cortex in unstimulated cells (Fig. 9 *A*). A low level of staining was also detected in the cytoplasm. HA-SYK also localized to the cell cortex when transiently expressed in 3T3 fibroblasts (Fig. 9 *B*). These data, therefore, indicated that the localization of SYK was similar to ZAP-70.

Discussion

This study demonstrates that ZAP-70 is localized at the cell cortex in a diffuse band under the plasma membrane in unstimulated Jurkat T cells. This specific targeting of ZAP-70 suggests that an anchoring site exists in the cell cortex, which concentrates it in this location. As ZAP-70 was also localized at the cell cortex when expressed in 3T3 fibroblasts, this putative anchoring site cannot be lymphoid cell specific. Comparison with phalloidin staining indicated that ZAP-70 distribution was distinct from that of cortical actin, suggesting that the actin cytoskeleton is unlikely to be its anchoring site (Figs. 5 *A* and 6, *A* and *B*). This conclusion was substantiated by the observation that the localization of ZAP-70 was unaffected by disruption of the actin cytoskeleton with cytochalasin D (Fig. 6 *C*). However, it is possible that ZAP-70 may interact with other components of the cortical cytoskeleton that are linked to the plasma membrane and, therefore, may be insensitive to the effects of cytochalasin D. Several examples exist of cortical cytoskeletal proteins which act as cross-linkers between the plasma membrane and the actin-based cytoskeleton. These include ankyrin, dystrophin, α-actinin, talin, and ERM family proteins such as ezrin (Hitt and Luna, 1994; Tsukita et al., 1997).

One noticeable feature of HA–ZAP-70 localization was its diffuse nature, which contrasted with the "tight" localization of LCK, which is directly bound to the plasma membrane via dual acylation of its NH_2 terminus (Koegl et al., 1994; Zlatkine et al., 1997). ZAP-70 was concentrated at the cell cortex in a gradient that reached a maximum coincident with the cortical cytoskeleton and plasma membrane, which could not be distinguished at the resolution of the confocal microscope. It is possible that the anchoring site

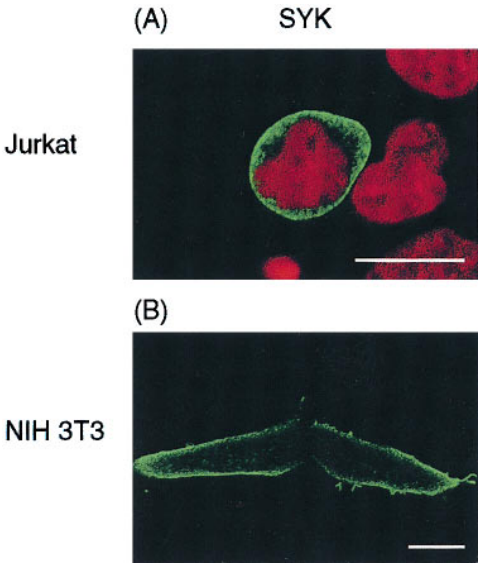


Figure 9. SYK localizes to the cell cortex. Jurkat T cells (*A*) or 3T3 fibroblasts (*B*) were transfected with a plasmid encoding NH_2 terminally HA-tagged SYK, fixed, and the localization of the expressed protein determined, as described in Fig. 7. Bars, 10 μm.

may have a similar distribution to ZAP-70. However, a more attractive model is that the putative anchoring molecule is located in the region where ZAP-70 staining was highest and the interaction with ZAP-70 is very weak and dynamic such that ZAP-70 is continually dissociating from the anchor site and diffusing back into the cytoplasm. Consistent with this hypothesis, cell fractionation experiments suggested that the interaction between ZAP-70 and its putative anchoring molecule, which would be predicted to be insoluble, was of low affinity such that this association was not stable to cell lysis. Cortactin, which is localized to the cortical actin cytoskeleton by binding directly to F-actin via an internal 37-amino acid repeat (Wu and Parsons, 1993), is also completely soluble after cell lysis (Wu et al., 1991). If Jurkat T cells were cultured for 48 h after transfection, rather than 24 h, HA–ZAP-70 was expressed at much higher levels and was then found throughout the cytoplasm (data not shown). This suggested that the putative anchor site in the cell cortex was saturable.

No gross changes in the localization of ZAP-70 were detected after stimulation of the cells with CD3 mAb. However, biochemical experiments have indicated that only ~1% of ZAP-70 becomes associated with the TCR after CD3 stimulation, which may be below the sensitivity of the immunofluorescence technique against the background of 99% which does not move (Weill et al., 1995). The data in this paper contrast with those in a recent study by Thome et al. (1995) in which an activation-inducible movement of ZAP-70 from the cytoplasm to CD3 caps was reported. However, those data imply that ZAP-70 quantitatively associates with the TCR after CD3 stimulation, which is unlikely based on the stoichiometry of association estimated from biochemical analyses (Weill et al., 1995). The reason for the discrepancies with this study is not clear.

Analysis of transfected mutant cDNAs indicated that ZAP-70 cortical targeting was not mediated by its tandem SH2 domains. Rather, distribution of the K369A mutant suggested that an active kinase domain was required for ZAP-70 localization. One model to account for this requirement is that autophosphorylation of ZAP-70, within either the kinase domain or hinge, may create binding sites for SH2-containing proteins which tether it in the cell cortex. It is unlikely that ZAP-70 is transphosphorylated by ZAP-70 to target it to the cell cortex, as kinase-inactive ZAP-70 was delocalized when expressed in Jurkat T cells which express endogenous ZAP-70 that is cortically localized and enzymatically active. Analysis of point mutants formally demonstrated that none of the known inducible sites of phosphorylation (Y292, Y492, Y493) were required for cortical targeting (Fig. 8) which was perhaps not surprising since ZAP-70 is cortically targeted in unstimulated Jurkat T cells. Thus, this model requires that ZAP-70 is autophosphorylated on other sites. Both wild-type ZAP-70 and the ΔSH2-ZAP-70 mutant, which were both targeted to the cell cortex, were tyrosine phosphorylated at only very low levels in unstimulated Jurkat T cells (Fig. 2 *E*; data not shown). The putative sites of basal autophosphorylation, therefore, would also have to turn over very rapidly. A more attractive model proposes that the K369A mutation, which renders ZAP-70 kinase inactive by preventing ATP binding (Johnson et al., 1996), is also unable to bind to one of its substrates due to the alteration of the catalytic binding site. In this model, therefore, ZAP-70 is localized by interaction with one of its substrates which is present in the cell cortex. Interestingly, PKC α is localized to focal contacts by interaction with proteins that it phosphorylates, which include vinculin, talin, and the MARCKS protein (Chapline et al., 1993; Hyatt et al., 1994; Liao et al., 1994). In this case, phosphorylation of these substrates abolishes interaction with PKC α, thereby allowing the enzyme to phosphorylate other proteins. The identification of the anchoring molecule or molecules for ZAP-70 will be important to understand why the K369A mutant is delocalized.

As discussed in the introduction, biochemical and genetic experiments have indicated that ZAP-70 is recruited to tyrosine-phosphorylated TCR subunits via its two SH2 domains. ZAP-70 is then itself tyrosine phosphorylated by LCK and activated. This has suggested a model in which ZAP-70 translocates from the cytoplasm to the plasma membrane to become activated (Weiss and Littman, 1994). The data in this paper indicate that ZAP-70 is already localized very close to the plasma membrane in unstimulated Jurkat T cells. However, ZAP-70 only becomes tyrosine phosphorylated and activated after CD3 mAb stimulation. Thus it is likely that, although ZAP-70 is close to or at the plasma membrane in unstimulated cells, it requires CD3 mAb stimulation to relocalize it to phospho-ITAMs, where it can then be phosphorylated by LCK. Consistent with this hypothesis, a transmembrane chimera of ZAP-70, which is anchored at the plasma membrane, is constitutively active when expressed in Jurkat T cells but requires functional LCK coexpression (Yamasaki et al., 1996). This raises the question of why ZAP-70 is localized to the cell cortex. One possibility is that this may increase the rapidity with which signaling can be initiated via the TCR by maintaining a high concentration of ZAP-70 that has to move only a short distance to interact with phospho-ITAMs and become activated. The major increase in tyrosine phosphorylation after TCR stimulation occurs at the cell cortex (Ley et al., 1994*a*). The targeting of ZAP-70, therefore, may also localize it close to its downstream targets. The similar localization of SYK to the cell cortex (Fig. 9) suggests that compartmentalization may play an analogous role in the function of this related protein tyrosine kinase.

Kong et al. (1996) have recently proposed a model for ZAP-70 activation in which it is first phosphorylated by LCK on Y493 after binding to phospho-ITAMs and then autophosphorylates on Y292 and Y492. This model is based on two observations. First, a kinase-inactive GST fusion protein of ZAP-70 is phosphorylated only on Y493 when mixed with the isolated kinase domain of LCK, and second, kinase-inactive ZAP-70 is phosphorylated at much lower levels compared with the wild-type protein after BCR stimulation, when expressed in SYK-negative DT40 B cells. However, the results in this paper suggest an alternative explanation for the latter result. Kinase-inactive ZAP-70 is delocalized from the cell cortex and presumably, therefore, can less efficiently interact with the BCR ITAMs and with PTKs at the plasma membrane. Thus the reduced phosphorylation of the kinase-inactive ZAP-70 in DT40 B cells, after BCR stimulation, may result from its inaccessibility to PTKs in the cell cortex which can transphosphory-

late it. Mege et al. (1996) have recently reported that kinase-dead ZAP-70 is constitutively associated with the phospho-ζ if sufficiently overexpressed in Jurkat T cells. These authors have suggested a model in which, under basal conditions, TCR ITAMs may be continually phosphorylated. However, the accumulation of phospho-ITAMs is prevented by the action of a phosphatase that rapidly dephosphorylates them. Overexpression of kinase-inactive ZAP-70 has been postulated to prevent phospho-ITAM dephosphorylation by its binding to them, resulting in their constitutive phosphorylation in unstimulated cells (Mege et al., 1996). Since kinase-inactive ZAP-70 is delocalized from the cell cortex, these results suggest that ZAP-70 localization may not be important for interaction with ITAMs if it is expressed at supraphysiological levels. However, it is likely that, at physiological levels, the targeting of ZAP-70 to the cell cortex will be necessary for its efficient association with the TCR and subsequent interaction with LCK and downstream targets.

Recent studies have indicated that phosphorylation events in a cell are not only controlled by the balance of kinase and phosphatase activity but also the subcellular location of these enzymes (Mochly-Rosen, 1995; Faux and Scott, 1996*b*). One emerging mechanism for such compartmentalization is the interaction of kinases and phosphatases with targeting proteins that localize enzymes close to their site of action (Faux and Scott, 1996*a*). This study demonstrates that both ZAP-70 and SYK are constitutively localized to the T cell cortex where they are in close proximity to the TCR with which they interact to become activated (Weiss and Littman, 1994; Chan and Shaw, 1996). These experiments raise the possibility that ZAP-70 and SYK may also be compartmentalized via interaction with a targeting protein or proteins. Recent studies from one of our laboratories have demonstrated that binding of LCK to the plasma membrane via dual acylation of its NH_2 terminus is essential for its function in TCR signaling (Kabouridis, P., A.I. Magee, and S.C. Ley, unpublished observations). It is anticipated that the constitutive cortical localization of ZAP-70 and SYK will likewise be important for both their activation and access to downstream targets, which previous studies have indicated are also concentrated in the cell cortex (Takagi et al., 1991; Ley et al., 1994*a*).

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References

Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell.* 70:751–763.

Baniyash, M., P. Garcia-Morales, E. Luong, L.E. Samelson, and R.D. Klausner. 1988. The T cell antigen receptor ζ chain is tyrosine phosphorylated upon activation. *J. Biol. Chem.* 263:18225–18230.

Bu, J.Y., A.S. Shaw, and A.C. Chan. 1995. Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl. Acad. Sci. USA.* 92:5106–5110.

Chan, A.C., and A.S. Shaw. 1996. Regulation of antigen receptor signal transduction by protein tyrosine kinases. *Current Opin. Immunol.* 8:394–401.

Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell.* 71: 649–662.

Chan, A.C., N.S. van Oers, A. Tran, L. Turka, C.L. Law, J.C. Ryan, E.A. Clark, and A. Weiss. 1994*a.* Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *J. Immunol.* 152:4758–4766.

Chan, T.A., C.A. Chu, K.A. Rauen, M. Kroiher, S.M. Tatarewicz, and R.E. Steele. 1994*b.* Identification of a gene encoding a novel protein-tyrosine kinase containing SH2 domains and ankyrin-like repeats. *Oncogene.* 9:1253–1259.

Chan, A.C., M. Dalton, R. Johnson, G.-H. Kong, T. Wang, R. Thoma, and T. Kurosaki. 1995. Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2499–2508.

Chapline, C., K. Ramsay, T. Klauck, and S. Jaken. 1993. Interaction cloning of protein kinase C substrates. *J. Biol. Chem.* 268:6858–6861.

Chu, D.H., H. Spits, J.-F. Peyron, R.B. Rowley, J.B. Bolen, and A. Weiss. 1996. The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signalling. *EMBO (Eur. Mol. Biol. Organ.) J.* 16: 6251–6261.

Clevers, H., B. Alarcon, T. Wileman, and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6:629–662.

Duplay, P., M. Thome, F. Herve, and O. Acuto. 1994. p56lck interacts via its src homology 2 domain with the ZAP-70 kinase. *J. Exp. Med.* 179:1163–1172.

Fargnoli, J., A.L. Burkhardt, M. Lavert, S.A. Kut, N.S.C. van Oers, A. Weiss, and J.B. Bolen. 1995. SYK mutation in Jurkat E6-derived clones results in lack of $p72^{syk}$ expression. *J. Biol. Chem.* 270:26533–26537.

Faux, M.C., and J.D. Scott. 1996*a.* Molecular glue: kinase anchoring and scaffold proteins. *Cell.* 85:9–12.

Faux, M.C., and J.D. Scott. 1996*b*. More on target with protein phosphorylation: conferring specificity by location. *Trends Biochem. Sci.* 21:312–315.

Groves, T., P. Smiley, M.P. Cooke, K. Forbush, R.M. Perlmutter, and C.J. Guidos. 1996. FYN can partially substitute for LCK in T cell development. *Immunity.* 5:417–428.

Hitt, A.L., and E.J. Luna. 1994. Membrane interactions with the actin cytoskeleton. *Curr. Opin. Cell Biol.* 6:120–130.

Hivroz, C., and A. Fischer. 1994. Immunodeficiency diseases. Multiple roles for ZAP-70. *Curr. Biol.* 4:731–733.

Hsi, E.D., J.N. Siegel, Y. Minami, E.T. Luong, R.D. Klausner, and L.E. Samelson. 1989. T cell activation induces rapid tyrosine phosphorylation of a limited number of cellular substrates. *J. Biol. Chem.* 264:10836–10842.

Huby, R.D.J., G.W. Carlile, and S.C. Ley. 1995. Interactions between the protein-tyrosine kinase, ZAP-70, the proto-oncoprotein Vav and tubulin in Jurkat T cells. *J. Biol. Chem.* 270:30241–30244.

Hyatt, S.L., L. Liao, A. Aderem, A.C. Nairn, and S. Jaken. 1994. Correlation between protein kinase C binding proteins and substrates in REF52 cells. *Cell Growth Differ.* 5:495–502.

Isakov, N., R.L. Wange, W.H. Burgess, J.D. Watts, R. Aebersold, and L.E. Samelson. 1995. ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity. *J. Exp. Med.* 181:375–380.

Iwashima, M., B.A. Irving, N.S. van Oers, A.C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science (Wash. DC).* 263:1136–1139.

Johnson, L.N., M.E.M. Noble, and D.J. Owen. 1996. Active and inactive protein kinases: structural basis for regulation. *Cell.* 85:149–158.

Kanner, S.B., A. Aruffo, and P.Y. Chan. 1994. Lymphocyte antigen receptor activation of a focal adhesion kinase-related tyrosine kinase substrate. *Proc. Natl. Acad. Sci. USA.* 91:10484–10487.

Karnitz, L., S.L. Sutor, T. Toshihiko, J.C. Reed, M.P. Bell, D.J. McKean, P.J. Leibson, and R.T. Abraham. 1992. Effects of p56lck deficiency on the growth and cytolytic effector function of an interleukin-2 dependent cytotoxic T cell line. *Mol. Cell. Biol.* 12:4521–4530.

Katzav, S., M. Sutherland, G. Packham, T. Yi, and A. Weiss. 1994. The protein tyrosine kinase ZAP-70 can associate with the SH2 domain of proto-Vav. *J. Biol. Chem.* 269:32579–32585.

Klausner, R.D., and L.E. Samelson. 1991. T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell.* 64:875–878.

Koegl, M., P. Zlatkine, S.C. Ley, S.A. Courtneidge, and A.I. Magee. 1994. Palmitoylation of multiple Src-family kinases at a homologous NH_2-terminal motif. *Biochem. J.* 303:749–753.

Kong, G.H., J.Y. Bu, T. Kurosaki, A.S. Shaw, and A.C. Chan. 1995. Reconstitution of SYK function by the ZAP-70 protein tyrosine kinase. *Immunity.* 2: 485–492.

Kong, G., M. Dalton, J. Bubeck-Wardenburg, D. Straus, T. Kurosaki, and A.C. Chan. 1996. Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. *Mol. Cell. Biol.* 16:5026–5035.

Law, C.-L., S.P. Sidorenko, K.A. Chandran, K.E. Draves, A.C. Chan, A. Weiss, S. Edelhoff, C.M. Disteche, and E.A. Clark. 1994. Molecular cloning of human SYK. *J. Biol. Chem.* 269:12310–12319.

Ley, S.C., M. Marsh, C.R. Bebbington, K. Proudfoot, and P. Jordan. 1994*a.* Distinct intracellular localization of Lck and Fyn protein tyrosine kinases in human T lymphocytes. *J. Cell Biol.* 125:639–649.

Ley, S.C., W. Verbi, D.J. Pappin, B. Druker, A.A. Davies, and M.J. Crumpton. 1994*b*. Tyrosine phosphorylation of alpha tubulin in human T lymphocytes. *Eur. J. Immunol.* 24:99–106.

Liao, L., S.L. Hyatt, C. Chapline, and S. Jaken. 1994. Protein kinase C domains involved in interactions with other proteins. *Biochemistry.* 33:1229–1233.

Mallick-Wood, C.A., W. Pao, A.M. Cheng, J.M. Lewis, S. Kulkarni, J.B. Bolen, B. Rowley, R.E. Tigelaar, T. Pawson, and A.C. Hayday. 1996. Disruption of epithelial γδ T cell repertoires by mutation of the SYK tyrosine kinase. *Proc. Natl. Acad. Sci. USA.* 93:9704–9709.

Mege, D., V.D. Bartolo, V. Germain, L. Tuosto, F. Michel, and O. Acuto. 1996. Mutation of tyrosines 492/493 in the kinase domain of ZAP-70 affects multiple T cell receptor signaling pathways. *J. Biol. Chem.* 271:32644–32652.

Mochly-Rosen, D.. 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science (Wash. DC).* 268:247–251.

Molina, T.J., K. Kishihara, D.P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C.J. Paige, K.U. Hartmann, A. Veillette, et al. 1992. Profound block in thymocyte development in mice lacking p56lck. *Nature (Lond.).* 357:161–164.

Negishi, I., N. Motoyama, K.-I. Nakayama, K. Nakayama, S. Senju, S. Hatakeyama, Q. Zhang, A.C. Chan, and D.Y. Loh. 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature (Lond.).* 376: 435–438.

Neumeister, E.N., Y. Zhu, S. Richard, C. Terhorst, A.C. Chan, and A.S. Shaw. 1995. Binding of ZAP-70 to phosphorylated T-cell receptor ζ and η enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. *Mol. Cell. Biol.* 15:3171–3178.

Qian, D., I. Griswold-Prenner, M.R. Rosner, and F.W. Fitch. 1993. Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation. *J. Biol. Chem.* 268:4488–4493.

Rowley, R.B., A.L. Burkardt, H.-G. Chao, G.R. Matsueda, and J.B. Bolen. 1995*a*. Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. *J. Biol. Chem.* 270:11590–11594.

Rowley, R.B., A.L. Burkhardt, H.-G. Chao, G.R. Matsueda, and J.B. Bolen. 1995*b*. SYK protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. *J. Biol. Chem.* 270:11590–11594.

Salmeron, A., T.B. Ahmad, G.W. Carlile, D. Pappin, R.P. Narsimhan, and S.C. Ley. 1996. Activation of MEK-1 and SEK-1 by TPL-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 15: 817–826.

Sancho, J., R. Franco, T. Chatila, C. Hall, and C. Terhorst. 1993. The T cell receptor associated CD3 ϵ protein is phosphorylated upon T cell activation in the two tyrosine residues of a conserved signal transduction motif. *Eur. J. Immunol.* 23:1636–1642.

Schneider, C., R.A. Newman, D.R. Sutherland, U. Asser, and M.F. Greaves. 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. *J. Biol. Chem.* 257:10766–10769.

Shiue, L., M.J. Zoller, and J.S. Brugge. 1995. Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs on the high affinity receptor for IgE. *J. Biol. Chem.* 270:10498–10502.

Stein, P.L., H.-M. Lee, S. Rich, and P. Soriano. 1992. pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell.* 70:741–750.

Straus, D.B., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell.* 70:585–593.

Straus, D.B., and A. Weiss. 1993. The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. *J. Exp. Med.* 178:1523–1530.

Takagi, S., M. Daibata, T.J. Last, R.E. Humphreys, D.C. Parker, and T. Sairenji. 1991. Intracellular localization of tyrosine kinase substrates beneath crosslinked surface immunoglobulins in B cells. *J. Exp. Med.* 174:381–388.

Taniguchi, T., T. Kobayashi, J. Kondo, K. Takahashi, H. Nakamura, J. Suzuki, K. Nagai, T. Yamada, S. Nakamura, and H. Yamamura. 1991. Molecular cloning of a porcine gene syk that encodes a 72 kDa protein tyrosine kinase showing high susceptibility to proteolysis. *J. Biol. Chem.* 266:15790–15796.

Thome, M., P. Duplay, M. Guttinger, and O. Acuto. 1995. Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T cell receptor/CD3/ζ complex. *J. Exp. Med.* 181:1997–2006.

Tsukita, S., S. Yonemura, and S. Tsukita. 1997. ERM (ezrin/radixin/moesin) family: from cytoskeleton to signal transduction. *Curr. Opin. Cell. Biol.* 9:70–75.

Turner, M., P.J. Mee, P.S. Costello, O. Williams, A.A. Price, L.P. Duddy, M.T. Furlong, R.L. Geahlen, and V.L.J. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase SYK. *Nature (Lond.).* 378:298–302.

van Oers, N.S.C., N. Killeen, and A. Weiss. 1994. ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR zeta in murine thymocytes and lymph node T cells. *Immunity.* 1:675–685.

van Oers, N.S.C., H. von Boehmer, and A. Weiss. 1995. The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-ζ subunit. *J. Exp. Med.* 182:1585–1590.

van Oers, N.S.C., N. Killeen, and A. Weiss. 1996*a*. Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. *J. Exp. Med.* 183:1053–1062.

van Oers, N.S.C., B. Lowin-Kropf, D. Finlay, K. Connolly, and A. Weiss. 1996*b*. αβ T cell development is abolished in mice lacking both LCK and FYN protein tyrosine kinase. *Immunity.* 5:429–436.

Wange, R.L., S.N. Malek, S. Desiderio, and L.E. Samelson. 1993. Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor zeta and CD3 epsilon from activated Jurkat T cells. *J. Biol. Chem.* 268:19797–19801.

Wange, R.L., R. Guitian, N. Isakov, J.D. Watts, R. Aebersold, and L.E. Samelson. 1995. Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. *J. Biol. Chem.* 270:18730–18733.

Watts, J.D., M. Affolter, D.L. Krebs, J.D. Wange, L.E. Samelson, and R. Aebersold. 1994. Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. *J. Biol. Chem.* 269:29520–29529.

Weill, R., J.-F. Cloutier, M. Fournel, and A. Veillette. 1995. Regulation of ZAP-70 by Src family tyrosine protein kinases in an antigen-specific T-cell line. *J. Biol. Chem.* 270:2791–2799.

Weiss, A. 1991. Molecular and genetic insights into T cell antigen receptor structure and function. *Annu. Rev. Genet.* 25:487–510.

Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell.* 73:209–212.

Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263–274.

Wiest, D.L., L. Yuan, J. Jefferson, P. Benveniste, M. Tsokos, R.D. Klausner, L.H. Glimcher, L.E. Samelson, and A. Singer. 1993. Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56lck tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. *J. Exp. Med.* 178:1701–1712.

Wiest, D.L., J.M. Ashe, R. Abe, J.B. Bolen, and A. Singer. 1996. TCR activation of ZAP-70 is impaired in CD4+CD8+ thymocytes as a consequence of intrathymic interactions that diminish available $p56^{lck}$. *Immunity.* 4:495–504.

Wu, H., and J.T. Parsons. 1993. Cortactin, an 80/85-kilodalton $pp60^{src}$ substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell. Biol.* 120:1417–1426.

Wu, H., A.B. Reynolds, S.B. Kanner, R.R. Vines, and J.T. Parsons. 1991. Identification and characterization of a novel cytoskeleton-associated $pp60^{src}$ substrate. *Mol. Cell. Biol.* 11:5113–5124.

Yamasaki, S., M. Takamatsu, and M. Iwashima. 1996. The kinase, SH3 and SH2 domains of LCK play critical roles in T-cell activation after ZAP-70 membrane localization. *Mol. Cell. Biol.* 16:7151–7160.

Zhao, Q., and A. Weiss. 1996. Enhancement of lymphocyte responsiveness by a gain-of-function mutation of ZAP-70. *Mol. Cell. Biol.* 16:6765–6774.

Zlatkine, P., B. Mehul, and A.I. Magee. 1997. Retargeting of cytosolic proteins to the plasma membrane by the LCK protein tyrosine kinase dual acylation motif. *J. Cell Science.* 110:673–679.