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# The Vav Binding Site (Y315) in ZAP-70 Is Critical for Antigen Receptor–mediated Signal Transduction

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## Summary

Stimulation of antigen receptors in T and B cells leads to the activation of the Src and Syk families of protein tyrosine kinases (PTK). These PTKs subsequently phosphorylate numerous intracellular substrates, including the 95-kD protooncogene product Vav. Vav is essential for both T and B cell development and T and B cell antigen receptor–mediated signal transduction. After receptor ligation, Vav associates with phosphorylated Syk and ZAP-70 PTKs, an interaction that depends upon its SH2 domain. Here we demonstrate that a point mutation of tyrosine 315 (Y315F) in ZAP-70, a putative Vav SH2 domain binding site, eliminated the Vav–ZAP-70 interaction. Moreover, the Y315 mutation impaired the function of ZAP-70 in antigen receptor signaling. Surprisingly, this mutation also resulted in marked reduction in the tyrosine phosphorylation of ZAP-70, Vav, SLP-76, and Shc. These data demonstrate that the Vav binding site in ZAP-70 plays a critical role in antigen receptor–mediated signal transduction.

S timulation of the TCR and B cell antigen receptors (BCR) initiates a cascade of signal transduction events involving the activation of two families of protein tyrosine kinases (PTKs), Src and Syk (1). The Src family members initiate these events by phosphorylating the tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) after TCR/BCR stimulation (1). The Syk and ZAP-70 PTKs are subsequently recruited to the phosphorylated ITAMs, where they become phosphorylated and activated (1). Activation of these kinases further leads to tyrosine phosphorylation of numerous cellular proteins including Vav, phospholipase C γ isoforms, Shc, and SLP-76 (1–4). Tyrosine phosphorylation and/or activation of these substrates ultimately results in downstream cytokine gene induction and other effector functions.

The protooncogene Vav is expressed exclusively in hematopoietic cells and contains an array of structural motifs, including a guanine nucleotide exchange (GEF) domain for the Rho/Rac/CDC42 family of small GTPases, a pleckstrin homology domain, and two src homology (SH) 3 domains that flank one SH2 domain (5, 6). Its homology to Dbl and CDC24 and recent functional data in vitro and in fibroblasts suggests that Vav functions as a GEF for the Rho/Rac/CDC42 family of small GTPases (5–8). Vav plays a critical role in lymphocyte development and activation, since T and B cell numbers are severely reduced in the ab-

sence of Vav (9–11). The small numbers of T and B cells which can develop in the absence of Vav display a profound and specific defect in TCR- and BCR-mediated signal transduction. Moreover, overexpression of Vav in Jurkat T cells results in a marked increase in basal nuclear factor of activated T cells (NFAT) or IL-2 promoter–driven transcriptional activity, which is further enhanced by TCR stimulation (12). However, the exact molecular mechanism by which Vav functions in lymphocytes remains to be determined.

We were interested in identifying upstream kinase(s) responsible for Vav tyrosine phosphorylation. We have previously shown that the Vav SH2 domain is required for its TCR/BCR-induced tyrosine phosphorylation (13). In addition, we and others have previously reported that tyrosine phosphorylated ZAP-70 can associate with the Vav SH2 domain after TCR stimulation (13–15). Interestingly, both ZAP-70 (Y315) and Syk (Y348) contain a consensus Vav SH2 domain binding sequence, YESP (16). By using the chicken B cell DT-40 in transient transfection experiments, we show here that Y315 in ZAP-70 is critical for antigen receptor–mediated signaling. We find that mutation of Y315 in ZAP-70 prevents its interaction with the Vav SH2 domain. The point mutation in ZAP-70 also results in global defects in antigen receptor–mediated signaling events, as measured by the marked reduction in inducible tyrosine phosphorylation of ZAP-70, Vav, SLP-76, and Shc. These data strongly suggest that Y315 of ZAP-70 plays a critical role in regulating ZAP-70 function.

## Materials and Methods

*DNA Constructs and Fusion Proteins.* The NFAT luciferase reporter construct was a gift from Dr. G. Crabtree (Stanford University, Stanford, CA). The Vav plasmid (pCI115) was constructed by subcloning human Vav into pCIneo (Invitrogen, San Diego, CA). The parental plasmid for the ZAP-70 mutant was pCDNA3-ZAP-70. The Y315F mutant of ZAP-70 (ZAP-70[Y315F]) was created by M13-based, oligonucleotide-directed, site-specific mutagenesis procedures (17). The myc epitope–tagged, wild-type ZAP-70 (pSXSRa-ZAP-myc) was provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD). DNA encoding wild-type rat Syk was subcloned into the mammalian expression vector pEFBOS. Glutathione S transferase (GST)-VavSH2 was provided by Dr. S. Katzav (Israel Air Force Aeromedical Center, Tel Hashomer, Israel). The human Shc plasmid and the FLAG epitope–tagged human SLP-76 cDNA were provided by Dr. M. Gishizky (Sugen Inc., Redwood City, CA) and Dr. G. Koretzky (University of Iowa, Iowa City, IA), respectively.

*Antibodies and Peptide.* The mAb used for the stimulation of the BCR was M4 (provided by Drs. M. Cooper and C.L. Chen, University of Alabama, Birmingham, AL). Anti-Vav polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti–ZAP-70 mAb (2F3.2) was described previously (18). The anti-myc epitope mAb (9E10) was provided by Dr. J.M. Bishop. The peptide used in this paper represents a biotinylated doubly phosphorylated version of the second ITAM of the TCR ζ chain (18).

*Cell Lines, Transfections, and Luciferase Assays.* Wild-type and various mutants of DT-40 cells were maintained and transfected transiently as previously described (17, 19). In brief, 30 μg of either an empty vector, wild-type ZAP-70 or ZAP-70(Y315F), and 20 μg of NFAT-Luc construct was used. Cells were then electroporated, processed, and assayed as described (17).

*Immunoprecipitations, Protein Precipitations, Peptide Binding, and Immunoblotting.* Cells were harvested, washed, were left either unstimulated or stimulated with M4 (2 μg/ml), and then lysed as previously described (13). Lysates were then immunoprecipitated with the indicated antibodies. When precipitated with GST fusion proteins, lysates were first precleared with GST alone (10 μg) before they were precipitated with the indicated GST fusion proteins (2–5 μg). Resulting immunoprecipitates or protein complexes were resolved by SDS-PAGE. Peptide binding and immunoblotting were carried out as previously described (18).

*In Vitro Kinase Assay.* After transient transfection, wild-type and mutant ZAP-70 were immunoprecipitated and in vitro kinase assays were performed as previously described (17). Samples were then analyzed by SDS-PAGE, transferred to polyvinylidene difluoride membrane, treated with 1 M KOH for 1 h, and then subjected to autoradiography and immunoblotting.

## Results and Discussion

*Mutation of Y315 Impairs ZAP-70 Function.* To examine functional requirements of Y315 in ZAP-70 in antigen receptor–mediated signal transduction, we transfected the Syk-deficient DT-40 B cells with either wild-type or the mutated ZAP-70 (ZAP-70[Y315F]) along with a NFAT reporter construct (19). Consistent with the previous reports (19, 20), loss of *syk* in DT-40 resulted in a complete block in BCR-stimulated NFAT activation, a defect that
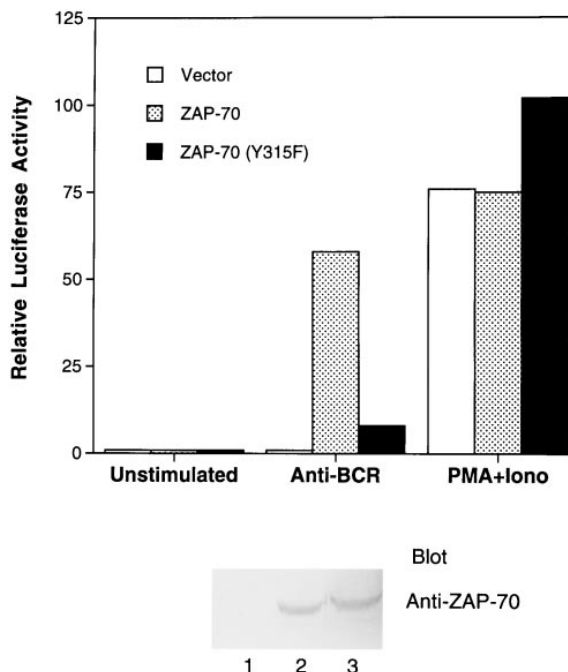


**Figure 1.** Mutation of Y315 in ZAP-70 impairs its function in BCR-mediated signal transduction. Syk-deficient DT-40 cells were transiently co-transfected with 20 μg of NFAT-Luc along with 30 μg of either an empty vector, wild-type ZAP-70, or ZAP-70(Y315F). After transfection (24–40 h), cells were either left unstimulated or stimulated with either anti-BCR (M4, 2 mg/ml) or PMA (50 ng/ml) plus ionomycin (1 μM) for 6–8 h, and subsequently assayed for luciferase activity. The results are shown as the fold induction of luciferase activity as compared with the activity in unstimulated cells transfected with the empty vector, which is ~200 arbitrary units. Luciferase activity was determined in triplicate in each experimental condition. The data are representative of at least three independent experiments. The lower panel represents anti–ZAP-70 blot (mAb 2F3.2) of equivalent amount of lysates from different transfectants in the luciferase assay described above.

could be rescued by expression of wild-type ZAP-70 (Fig. 1). In contrast, mutation of Y315 in ZAP-70 markedly impaired its ability to reconstitute BCR-induced NFAT activation (Fig. 1).

*Y315 of ZAP-70 Is Required for Interaction with the SH2 Domain of Vav.* To determine whether tyrosine 315 within the YESP motif of ZAP-70 functions as the Vav binding site, we transiently transfected Syk-deficient DT-40 cells with either wild-type ZAP-70 or ZAP-70(Y315F) and examined their ability to interact with a GST fusion protein containing the Vav SH2 domain (GST-VavSH2). As shown in Fig. 2 *A,* GST-VavSH2 fusion protein selectively bound to wild-type ZAP-70 after BCR stimulation or by treatment with the protein tyrosine phosphatase inhibitor pervanadate (Fig. 2 *A*). In contrast, mutation of Y315 in ZAP-70 markedly impaired its ability to bind to the Vav SH2 domain.

Lck also associates with ZAP-70 via its SH2 domain after TCR stimulation (21, 22). Interestingly, both wild-type ZAP-70 and ZAP-70(Y315F) from either BCR-stimulated or pervanadate-treated lysates could bind efficiently to the
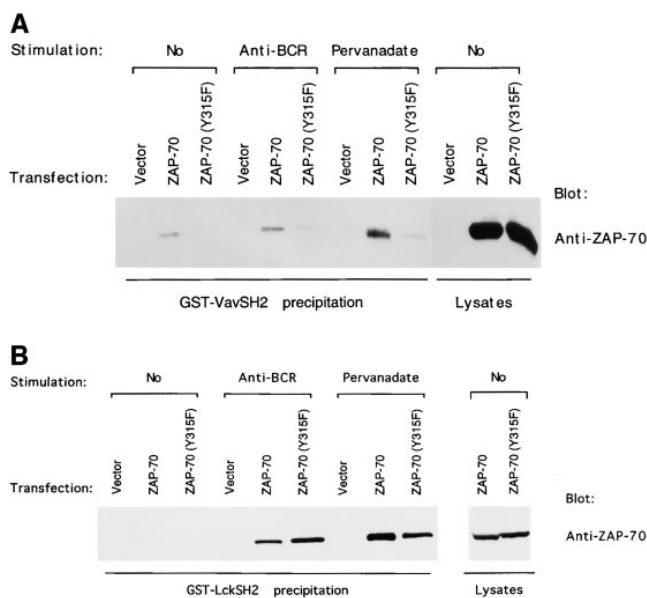
**Figure 2.** Y315 in ZAP-70 is required for its binding to the Vav SH2 domain. (*A*) Syk-deficient DT-40 cells were transiently transfected with either an empty vector, wild-type ZAP-70, or ZAP-70(Y315F). Cells were then either left unstimulated or stimulated with either anti-BCR or pervanadate, and lysed. The lysates were first precleared with GST alone and then mixed with a GST fusion protein containing the Vav SH2 domain. The protein complexes were blotted with anti–ZAP-70 mAb (2F3.2). The lysate lanes represent approximately one tenth volume of the total cell lysates used for GST precipitations. (*B*) Mutation of Y315 in ZAP-70 did not affect its ability to interact with the Lck SH2 domain. Syk-deficient cells were transfected as described in *A* and were either left unstimulated or stimulated with anti-BCR or pervanadate. The lysates were first precleared with GST alone and then precipitated with GST fusion protein containing the Lck SH2 domain. The protein complexes were then blotted with anti–ZAP-70 mAb (2F3.2).

GST-LckSH2 domain (Fig. 2 *B*), indicating that Y315 in ZAP-70 is specifically required for its interaction with the Vav SH2 domain, but not Lck SH2 domain.

Although initial phosphopeptide mapping studies failed to identify Y315 as one of the major tyrosine phosphorylated residues in ZAP-70, it is important to note that not all of the phosphorylation sites observed by two-dimensional peptide mapping were identified (23, 24). In fact, the corresponding residue (Y348) in Syk has been shown to be a major in vitro autophosphorylation site and it serves as the binding site for the Vav SH2 domain (15, 25). Moreover, not only did mutation of Y315 in ZAP-70 abolish the Vav–ZAP-70 interaction, this interaction could also be completely disrupted by the presence of a ZAP-70 peptide encompassing phosphorylated Y315 (14). These observations strongly argue that Y315 in ZAP-70 does represent an in vivo phosphorylation site after antigen receptor stimulation.

*Mutation of Y315 in ZAP-70 Markedly Reduces Tyrosine Phosphorylation of Vav, SLP-76, Shc, and ZAP-70 Itself.* To assess whether the Y315 mutation affects ZAP-70–mediated Vav tyrosine phosphorylation, we transiently coexpressed human Vav with empty vector, wild-type ZAP-70, ZAP-70(Y315F), or wild-type Syk into Lyn and Syk double-deficient DT-40 cells, in which BCR-induced Vav phos-

phorylation was completely absent (Fig. 3 *A*, data not shown, and reference 26). Coexpression of Vav with either wild-type ZAP-70 or Syk, but not ZAP-70(Y315F), led to Vav tyrosine phosphorylation, which was further induced by BCR stimulation (Fig. 3 *A*).

To further examine the impact of Y315 mutation on ZAP-70–mediated tyrosine phosphorylation of other downstream substrates, we analyzed the tyrosine phosphorylation status of SLP-76 and Shc. Coexpression of wild-type ZAP-70 with either SLP-76 or Shc in Syk-deficient or Lyn and Syk double-deficient DT-40 cells resulted in BCR-stimulated SLP-76 or Shc phosphorylation (Fig. 3, *B* and *C*, and data not shown). Surprisingly, mutation of Y315 in ZAP-70 substantially impaired its ability to mediate phosphorylation of SLP-76 and Shc (Fig. 3, *B* and *C*, and data not shown). In addition, mutation of Y315 also markedly reduced ZAP-70 tyrosine phosphorylation after antigen receptor stimulation in Syk-deficient cells and in Jurkat T cells (Fig. 3 *D* and data not shown). Taken together, Y315 of ZAP-70 is not only required for Vav tyrosine phosphorylation, but also for tyrosine phosphorylation of other downstream substrates such as SLP-76, Shc, and even for ZAP-70 itself.

*Mutation of Y315 in ZAP-70 Does Not Affect ZAP-70 Kinase Activity or Binding of ZAP-70 to Receptor ITAMs.* One explanation for the global defects of ZAP-70(Y315F) could be that the Y315F mutation reduced ZAP-70 kinase activity. Myc epitope–tagged ZAP-70 or ZAP-70(Y315F) was expressed in Lyn and Syk double-deficient cells and the kinase activity of anti-myc epitope–tagged immunoprecipitates was measured as both autophosphorylation and phosphorylation of an exogenous substrate, band III. The in vitro kinase assay failed to reveal a substantial difference between wild-type ZAP-70 and ZAP-70(Y315F) in their abilities to phosphorylate band III, although there may be a modest reduction in autophosphorylation of ZAP-70(Y315F) (Fig. 4 *A*).

Another critical step for ZAP-70 phosphorylation and activation is binding of ZAP-70 to the ITAMs after receptor stimulation. We used a biotinylated doubly phosphorylated ITAM peptide to precipitate ZAP-70 from lysates of Syk-deficient DT-40 cells transfected with either wild-type ZAP-70 or ZAP-70(Y315F). Similar amounts of wild-type ZAP-70 and ZAP-70(Y315F) bound to the phosphorylated peptide (Fig. 4 *B*). In addition, similar amounts of tyrosine-phosphorylated TCR ζ chains were found to co-immunoprecipitate with either form of ZAP-70 when analyzed in Jurkat T cells (data not shown). Taken together, these data demonstrate that mutation of Y315 in ZAP-70 did not dramatically affect its kinase activity or its binding to receptor ITAMs.

In summary, we demonstrate here that Y315 in ZAP-70 is required to interact with the Vav SH2 domain, and is critical for ZAP-70–mediated gene activation. Notably, the Y315-homologous residue in Syk is also required for its interaction with the Vav SH2 domain and for Vav phosphorylation (15). We provide evidence here that the Y315 mutation results in a global defect in ZAP-70–mediated signaling pathways, suggesting an important role of Y315 in regulating ZAP-70 function.
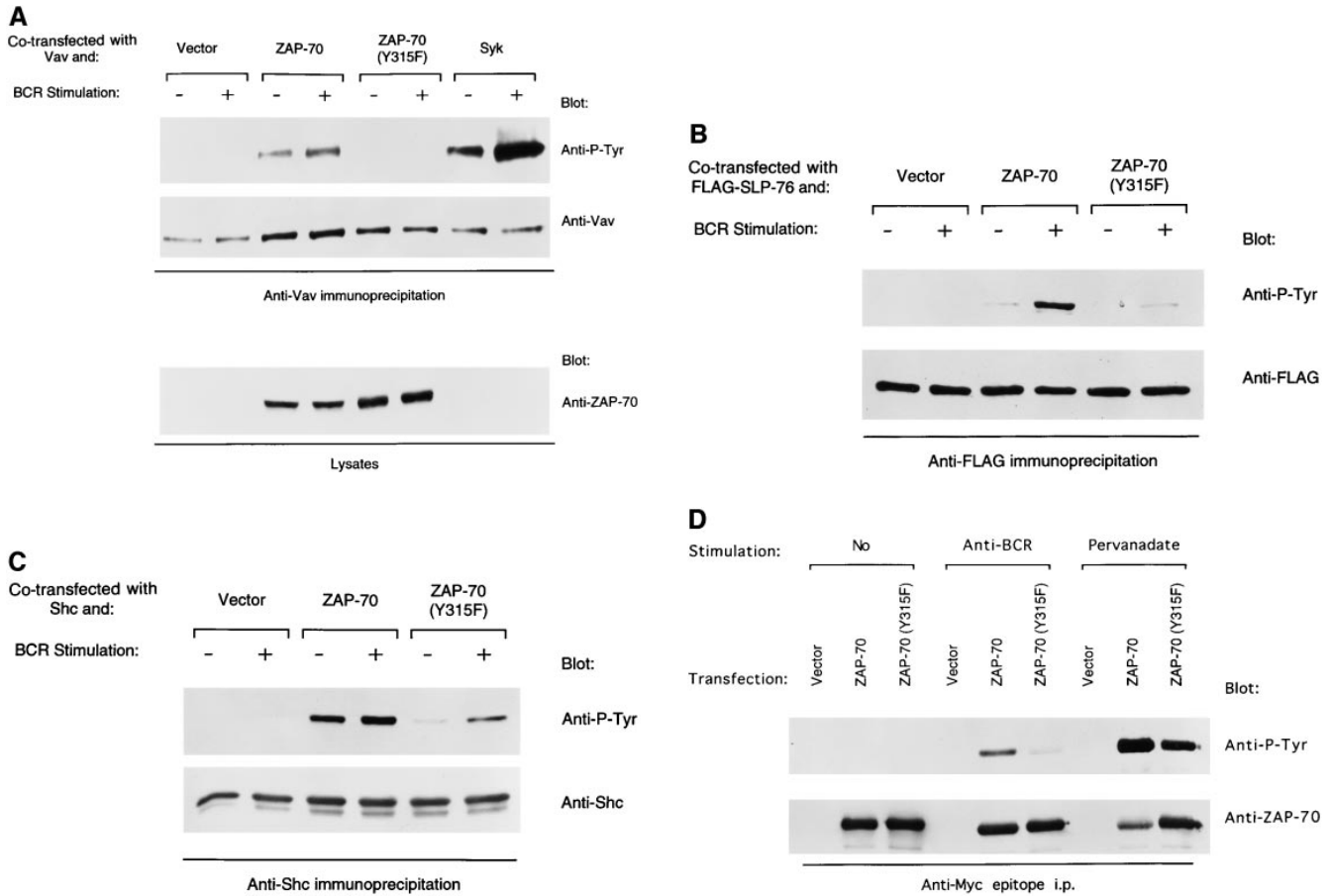
**Figure 3.** (*A*) Vav is not phosphorylated in cells transfected with ZAP-70(Y315F). Lyn/Syk double-deficient DT-40 cells were transiently co-transfected with human Vav (pCI115) along with either an empty vector, wild-type ZAP-70, ZAP-70(Y315F), or Syk. After transfection (20–40 h), cells were either left unstimulated or stimulated with anti-BCR (M4, 2 μg/ml) for 2 min, and then lysed. The lysates were immunoprecipitated with anti-Vav polyclonal Ab and the immune complexes were blotted with antiphosphotyrosine Ab (4G10; *top*). The blot was then stripped and reblotted with anti–Vav polyclonal Ab (*middle*). Equivalent amount of lysates were taken from each experimental condition and resolved on a SDS-PAGE and then blotted with anti–ZAP-70 mAb (2F3.2; *bottom*). (*B*) Mutation of Y315 in ZAP-70 reduces ZAP-70–mediated SLP-76 tyrosine phosphorylation. Syk-deficient DT-40 cells were transiently transfected with FLAG epitope–tagged human SLP-76 (*FLAG-SLP-76*) along with either an empty vector, wild-type ZAP-70, or ZAP-70(Y315F). Cells were stimulated and lysed as described in *A*. The lysates were immunoprecipitated with anti-FLAG epitope antibody (M2) and the immune complexes were blotted with 4G10 (*top*). The blot was then stripped and reblotted with anti-FLAG antibody (*bottom*). Anti–ZAP-70 Western blot revealed equivalent expression between wild-type ZAP-70 and ZAP-70 (Y315F) (data not shown). (*C*) Mutation of Y315 in ZAP-70 also reduces ZAP-70–mediated Shc tyrosine phosphorylation. Lyn/Syk double-deficient DT-40 cells were transiently transfected with human Shc cDNA along with either an empty vector, wild-type ZAP-70, or ZAP-70 (Y315F). Cells were stimulated and lysed as described in *A*. The lysates were immunoprecipitated with anti-Shc mAb and the immune complexes were blotted with 4G10 (*top*). The blot was then stripped and reblotted with polyclonal anti-Shc antibody (*bottom*). Anti–ZAP-70 Western blot showed equivalent expression between wild-type ZAP-70 and ZAP-70(Y315F) (data not shown). (*D*) Mutation of Y315 reduces BCR-mediated ZAP-70 tyrosine phosphorylation. Syk-deficient DT-40 cells were transiently transfected with either a vector, a myc epitope–tagged wild type or ZAP-70(Y315F). Cells were either left unstimulated or stimulated with anti-BCR or pervanadate for 2 min, and then lysed. The lysates were then immunoprecipitated with anti-myc antibody (9E10) and the immune complexes were blotted with 4G10 (*top*). The blot was then stripped and reblotted with anti–ZAP-70 mAb (2F3.2) (*bottom*).

Antigen receptor stimulation results in the assembly of multiprotein complexes, a process likely to facilitate efficient tyrosine phosphorylation and/or activation of appropriate signaling molecules (2). The Vav–ZAP-70 binding via Y315 may be important in initiating the proper formation of such signaling complexes, as both proteins are able to interact with many other signaling molecules (2). Since Vav possesses a GEF domain for Rho/Rac/CDC42 (7, 8),

the interaction between Vav and ZAP-70 may provide a mechanism by which ZAP-70 activates downstream Rho/Rac/CDC42-mediated signaling events such as cytoskeletal rearrangement. Mutation of Y315 in ZAP-70 may result in an impaired recruitment, phosphorylation and/or activation of many proteins including ZAP-70, Vav, SLP-76, and Shc.
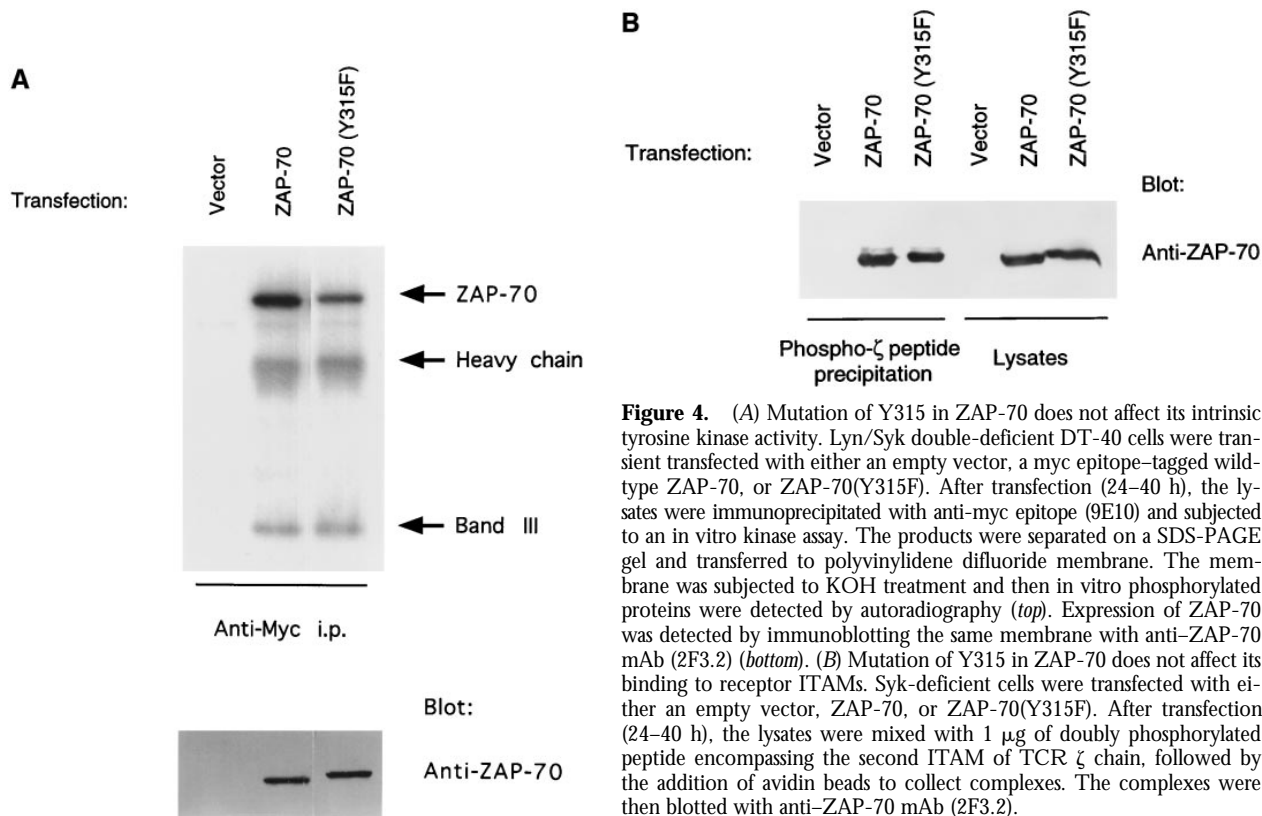
**A**

Transfection:



ZAP-70 ←

Heavy chain ←

Band III ←

Anti-Myc i.p.

Blot:

Anti-ZAP-70

**B**

Transfection:



Blot:

Anti-ZAP-70

Phospho-ζ peptide precipitation    Lysates

**Figure 4.** (*A*) Mutation of Y315 in ZAP-70 does not affect its intrinsic tyrosine kinase activity. Lyn/Syk double-deficient DT-40 cells were transient transfected with either an empty vector, a myc epitope–tagged wild-type ZAP-70, or ZAP-70(Y315F). After transfection (24–40 h), the lysates were immunoprecipitated with anti-myc epitope (9E10) and subjected to an in vitro kinase assay. The products were separated on a SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. The membrane was subjected to KOH treatment and then in vitro phosphorylated proteins were detected by autoradiography (*top*). Expression of ZAP-70 was detected by immunoblotting the same membrane with anti–ZAP-70 mAb (2F3.2) (*bottom*). (*B*) Mutation of Y315 in ZAP-70 does not affect its binding to receptor ITAMs. Syk-deficient cells were transfected with either an empty vector, ZAP-70, or ZAP-70(Y315F). After transfection (24–40 h), the lysates were mixed with 1 μg of doubly phosphorylated peptide encompassing the second ITAM of TCR ζ chain, followed by the addition of avidin beads to collect complexes. The complexes were then blotted with anti–ZAP-70 mAb (2F3.2).

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