

# T Cell Development and T Cell Responses in Mice with Mutations Affecting Tyrosines 292 or 315 of the ZAP-70 Protein Tyrosine Kinase

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## Abstract

After stimulation of the T cell receptor (TCR), the tyrosine residues 292 and 315 in interdomain B of the protein tyrosine kinase ZAP-70 become phosphorylated and plausibly function as docking sites for Cbl and Vav1, respectively. The two latter proteins have been suggested to serve as substrates for ZAP-70 and to fine-tune its function. To address the role of these residues in T cell development and in the function of primary T cells, we have generated mice that express ZAP-70 molecules with Tyr to Phe substitution at position 292 (Y292F) or 315 (Y315F). When analyzed in a sensitized TCR transgenic background, the ZAP-70 Y315F mutation reduced the rate of positive selection and delayed the occurrence of negative selection. Furthermore, this mutation unexpectedly affected the constitutive levels of the CD3- $\zeta$  p21 phosphoisoform. Conversely, the ZAP-70 Y292F mutation upregulated proximal events in TCR signaling and allowed more T cells to produce interleukin 2 and interferon  $\gamma$  in response to a given dose of antigen. The observation that ZAP-70 Y292F T cells have a slower rate of ligand-induced TCR downmodulation suggests that Y292 is likely involved in regulating the duration activated TCR reside at the cell surface. Furthermore, we showed that Y292 and Y315 are dispensable for the TCR-induced tyrosine phosphorylation of Cbl and Vav1, respectively. Therefore, other molecules present in the TCR signaling cassette act as additional adaptors for Cbl and Vav1. The present in vivo analyses extend previous data based on transformed T cell lines and suggest that residue Y292 plays a role in attenuation of TCR signaling, whereas residue Y315 enhances ZAP-70 function.

Key words: gene knockin • T cell antigen receptor • Vav1 • Cbl • signal transduction

## Introduction

The CD3 subunits of the TCR contain conserved cytoplasmic sequences termed the immunoreceptor tyrosine-based activation motifs (ITAMs).<sup>\*</sup> To exert their signaling func-

tions, ITAMs cooperate in a sequential manner with nonreceptor protein tyrosine kinases (PTKs) belonging to the Src and to the Syk/ZAP-70 PTK families (for a review, see reference 1). Two members of the Src family (Lck and to a lesser degree Fyn) initiate T cell activation by phosphorylating the tyrosine residues located within each CD3 ITAM and permitting the recruitment of ZAP-70 to the vicinity of the activated TCR. Next, by phosphorylating a tyrosine residue (Y493) located in the activation loop of the ZAP-70 catalytic domain, Lck increases the intrinsic kinase activity of the ITAM-bound ZAP-70 molecules, which in turn

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<sup>\*</sup>Abbreviations used in this paper: DN, double negative; DP, double positive; ES, embryonic stem; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; PTK, protein tyrosine kinase; SH, src homology; SP, single positive.

phosphorylate the adaptors LAT and SLP-76 and possibly other signaling proteins including Vav1, Cbl, and phospholipase C (PLC) $\gamma$ -1. Therefore, ZAP-70 does constitute a key player in the TCR signaling cassette and its ablation blocks mouse intrathymic T cell development at the double positive (DP) to single positive (SP) transition (2, 3).

Like Syk, ZAP-70 is composed of two tandemly arranged src homology (SH)2 domains which mediate its association with phosphorylated ITAMs and of a COOH-terminal tyrosine kinase domain. A linker region (interdomain B) located between the SH2 domains and the kinase domain, contains three tyrosine residues at position 292, 315, and 319. After TCR stimulation, each of these residues is phosphorylated (4, 5). Studies of recombinant ZAP-70 molecules in which tyrosines 292, 315, and 319 were mutated to phenylalanine, indicated that these residues are not directly involved in the regulation of ZAP-70 catalytic activity (6), but rather act in trans by specifically recruiting SH2 domain-containing molecules that regulate ZAP-70 activity and/or serve as substrate for ZAP-70. For instance, ZAP-70 molecules with a Tyr 319 to Phe mutation (ZAP-70 Y319F) fail to couple TCR ligation to downstream signaling pathways in both Jurkat T cells and a ZAP-70-deficient Jurkat derivative (7, 8). These observations correlate with the inability of the ZAP-70 Y319F molecules to recruit Lck and to upregulate their catalytic activity after TCR cross-linking (9). Once phosphorylated, Tyr 315 may also contribute to TCR-induced signaling by serving as an inducible binding site for the SH2 domain of Vav1, a Rho family GDP/GTP exchange factor. However, analysis of the functional consequence of the ZAP-70 Y315F mutation has led to conflicting data, that may reflect variations in the recipient cell lines used for gene transfer (7, 8, 10, 11). When expressed in Jurkat T cells, a ZAP-70 Y292F mutant resulted in the constitutive expression of a NF-AT-driven reporter gene (12, 13). Therefore, in contrast to Y315 and Y319, Y292 appears to exert a negative regulatory effect on ZAP-70. The atypical SH2 domain found in the adaptor Cbl binds to activated ZAP-70 via a motif that involves phosphotyrosine 292, and to interdomain B of Syk via an orthologous docking site (14, 15). Prevention of the phosphotyrosine-dependent interaction between Cbl and ZAP-70/Syk PTKs showed that Cbl is likely to be the protein that attenuates signaling by activated ZAP-70 and Syk kinases (16–20). When expressed into ZAP-70-deficient Jurkat T cells, ZAP-70 mutant molecules that lack most of interdomain B showed a reduced kinase activity but were still capable of reconstituting the TCR-driven induction of multiple reporter genes (21). This paradoxical result may be accounted for by the fact that this deletion simultaneously removed one negative regulatory site (Y292) and two positive regulatory sites (Y315 and Y319), and resulted in a compound phenotype. The above experiments, aiming at assessing the biological activity of mutant ZAP-70 molecules that fail to interact with defined cytoplasmic effectors/adaptors, have mainly been performed with transformed lymphoid cell lines in which some regulatory

pathways are profoundly disturbed (22). To define in vivo the role of the tyrosines found in the ZAP-70 interdomain B, we have generated knockin mice that express ZAP-70 molecules with a point mutation at position Y292 or Y315, and determined their effects on T cell development and on the function of ex vivo T cells challenged with physiologic amounts of antigen.

## Materials and Methods

### Mice

The P14 (line 327, reference 23) and H-Y (24, 25)  $\alpha\beta$  TCR transgenic lines were maintained on a C57BL/6 background. CD3 $\epsilon$ -deficient mice were as described previously (CD3 $\epsilon^{\Delta 5/\Delta 5}$ , reference 26).

### Vector Construction

ZAP-70 genomic clones were isolated from a 129/Ola phage library. After establishing the partial exon-intron structure of the ZAP-70 gene, the nucleotide sequence coding for interdomain B was determined and the tyrosine residue found at position 292 and 315 separately mutated to phenylalanine as described below.

### ZAP-70 Y292F Mutation

Mutagenesis was performed on a 170-bp Hinc II fragment encompassing exon 6. The mutated fragment was exchanged for the wild-type Hinc II fragment contained in a 2.1-kb Bam HI-Sma I restriction fragment subclone, and a diagnostic Cla I site and a loxP-flanked neo<sup>r</sup> gene introduced in the intron 3' of exon 6. Finally, the targeting construct was extended to give 3.5 kb and 3.0 kb of homologous sequences 5' and 3' of the neo<sup>r</sup> gene, respectively (see Fig. 1 A). After electroporation of E14 129/Ola embryonic stem (ES) cells and selection in G418, colonies were screened for homologous recombination by Southern blot analysis using 5' and 3' single-copy probes. The 5' probe is a 329-bp Sac II-Sma I fragment isolated from a ZAP-70 cDNA and the 3' probe is a 1.1-kb KpnI-EcoRI fragment isolated from a ZAP-70 genomic clone. When tested on Xba I-digested DNA, the 3' probe hybridizes either to a 7.0-kb wild-type fragment or to a 6.2-kb recombinant fragment. On Eco RI-digested DNA, the 5' probe hybridizes either to a 13.5-kb wild-type fragment or to a 10.6-kb recombinant fragment. Homologous recombinant ES clones were further checked for the presence of the intended mutation by sequencing the genomic segment corresponding to exon 6. Finally, a neo probe was used to ensure that adventitious nonhomologous recombination events had not occurred in the selected clones.

### ZAP-70 Y315F Mutation

Mutagenesis was performed on a 220-bp Sma I-SphI fragment containing the relevant segment of exon 7. A diagnostic Sac I restriction enzyme cleavage site was introduced into exon 7 to facilitate the subsequent identification of the ZAP-70 Y315F mutant mice. This modification does not modify the resulting ZAP-70 amino acid sequence with the exception of the Y315F mutation. A loxP-flanked neo<sup>r</sup> gene was additionally introduced in the intron 5' to exon 3. The final targeting construct contained 1.6 kb and 5 kb of homologous sequences 5' and 3' of the neo<sup>r</sup> gene, respectively (Fig. 1 B). After electroporation and selection in G418, recombinant colonies were identified by Southern blot

analysis using probes identical to those used for screening the ZAP-70 Y292F recombinant colonies (see above). In the case of the ZAP-70 Y315F mutation, the 3' probe hybridizes either to a 7.0-kb wild-type fragment or to a 8.0-kb recombinant fragment, whereas the 5' probe hybridizes either to a 13.5-kb wild-type fragment or to a 8.6-kb recombinant fragment. Homologous recombinant clones were confirmed via XbaI-SacI double digest and determination of the genomic sequence corresponding to exon 7. A neo probe was used to ensure that adventitious recombination events had not occurred in the selected clone.

#### Production of Mutant Mice

Mutant ES cells were injected into Balb/c blastocysts. One ZAP-70 Y292F and one ZAP-70 Y315F recombinant ES clones were found capable of germline transmission. The neo<sup>r</sup> gene flanking the ZAP-70 Y292F and ZAP-70 Y315F mutant loci was removed by crossing the chimeric mice to the Deleter mice (27). Mice with deletion of the loxP-flanked neo<sup>r</sup> gene were intercrossed to produce homozygous mutant mice. Screening of mice for the presence of the intended ZAP-70 mutations was performed by PCR using the following pairs of oligonucleotides: Y292F a: 5'-ATGGAGGAGATGGCCATGCAGGGA-3', and Y292F b: 5'-GCATGGACAGACCCCTGGC-3'; Y315F c: 5'-TGGGAAAGCCACTGCTGGATGTC-3', and Y315F d: 5'-TGTGTGCTGGATGTAGGACCCAGG-3'. Most of the studies were performed after backcrossing the mutant mice on a C57BL/6 background for at least five generations.

#### Flow Cytometric Analysis

Flow cytometric analysis was performed as described previously (28). Antibodies against CD3 $\epsilon$  (2C11), CD4 (GK1.5), CD8- $\alpha$  (56-6.7), V $\alpha$ 2 (B20.1), CD25 (7D4), CD69 (H1.2F3), and CD5 (53-7.5) were purchased from BD PharMingen. The biotinylated T3.70 anticolonotypic antibody (25) was a gift of H.T. He and A.M. Bernard, Centre d'Immunologie de Marseille-Luminy, Marseilles, France.

#### Antibodies

The following antibodies were used for immunoprecipitations and Western blot analysis: anti-CD3  $\zeta$  chain antiserum 873 (raised against a peptide corresponding to residues 1–11 of the human  $\zeta$  protein; 29), anti-ZAP-70 antiserum 50 (a gift of Dr. J.-M. Rojo, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain), anti-Cbl (C-15) antiserum (purchased from Santa Cruz Biotechnology, Inc.), anti-SLP-76 (a gift from Dr. G. Koretzky, University of Pennsylvania, Philadelphia, PA), anti-Vav1, and anti-LAT antisera were purchased from Upstate Biotechnology. The anti-Vav1 monoclonal antibody Vav-30 was a gift of Dr. J. Griffin (Dana Farber Cancer Institute, Boston, MA), the anti-ZAP-70 monoclonal antibody clone 29 was purchased from Transduction Laboratories, and the anti-PLC $\gamma$ -1 and the 4G10 antiphosphotyrosine monoclonal antibodies purchased from Upstate Biotechnology.

#### Immunoprecipitation, SDS-PAGE, and Immunoblotting

Thymocytes or lymph node cells were incubated for 20 min on ice with 20  $\mu$ g/ml anti-CD3 Ab (2C11), and the bound antibodies subsequently cross-linked with 20  $\mu$ g/ml of goat anti-hamster IgG for 3 min at 37°C. Cells were then lysed with 1% NP40-containing lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM magnesium chloride, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10  $\mu$ g/ml leupeptin, 10

$\mu$ g/ml aprotinin, 1 mM Pefabloc-SC, and 50 mM sodium orthovanadate). Immunoprecipitation, immunoblotting, and detection of proteins by enhanced chemiluminescence (Amersham Pharmacia Biotech) were performed as described previously (30).

#### Purification of CD8<sup>+</sup> T Cells from Lymph Nodes

Lymph node cells were incubated with a cocktail of antibodies directed against CD4 (H129.19.6), B220 (RA.36.B2), and MHC class II (M5/114) molecules. Cells reacting with at least one of the above antibodies were removed with Dynabeads M450 precoated with sheep anti-rat IgG. Purity of the remaining cells was controlled by flow cytometry. In all instances, this purification protocol resulted in cell suspensions containing 96–98% CD8<sup>+</sup> T cells.

#### Intracellular Staining

**Cytokines.** CD8<sup>+</sup> T cells ( $0.5 \times 10^6$ ) purified from lymph nodes from P14 TCR transgenic mice were cultured in 24-well plates in the presence of CD3 $\epsilon$ -deficient spleen cells ( $3 \times 10^6$ ) that were prepulsed for 2 h with the p33 agonist peptide ( $10^{-8}$  M). After 0, 4, 19, 39, and 63 h, cells were collected, and counted. Before intracellular cytokine staining, cells ( $1.5 \times 10^6$ ) were cultured for 4 h in the presence of monensin (GolgiStop; BD PharMingen) at a final concentration of 2  $\mu$ M. For staining, cells were immediately placed on ice, washed, resuspended in PBS 1 $\times$ , 1% FCS, 0.09% sodium azide, and stained with an APC-conjugated anti-CD8 antibody. To monitor intracellular cytokines, cells were then fixed using the cytofix/cytoperm kit (BD PharMingen), according to the manufacturer's instructions. Each cell sample was subsequently splitted into two aliquots; one of them was stained with a combination of FITC-conjugated anti-IFN- $\gamma$  and PE-conjugated anti-IL-2 antibodies, and the other stained with a combination of fluorochrome-conjugated and isotype-matched negative control Ig (BD PharMingen). After a final wash, CD8<sup>+</sup> cells ( $10^4$ ) were analyzed on a FACSCalibur™ flow cytometer after gating out dead cells using forward and side scatters.

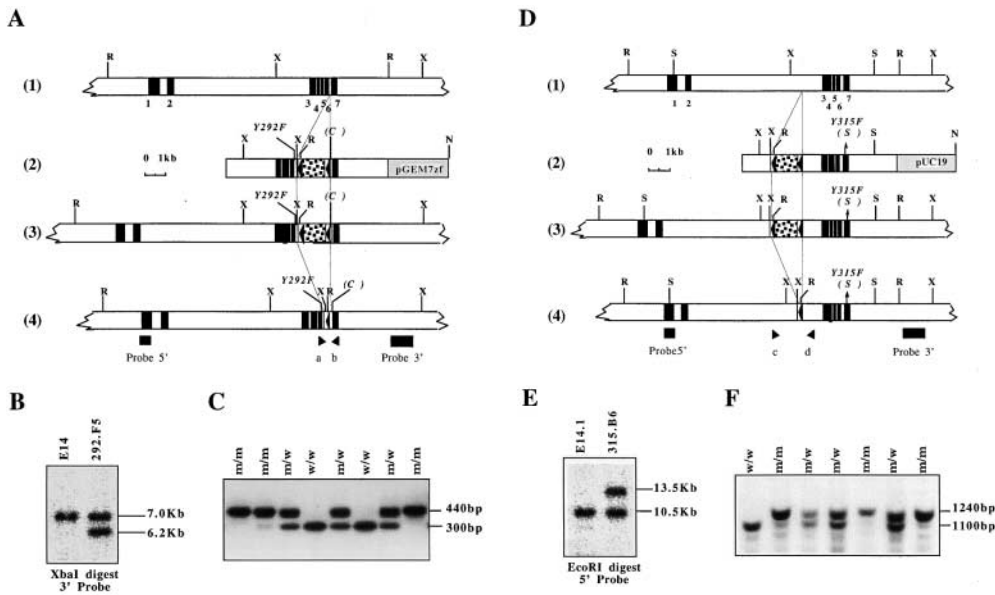
**Syk.** Cells were stained for the presence of Syk using the 5F5.2 anti-Syk monoclonal antibody (31).

#### TCR Downregulation and Induction of Cell-Surface Molecules

Lymph node T cells were cultured with peptide-loaded RMA-S cells (32). After incubation at 37°C for the specified time, cells were resuspended vigorously in PBS containing 0.5 mM EDTA and stained with antibody combinations allowing to gate on CD8<sup>+</sup> T cells and to simultaneously determine the levels of V $\alpha$ 2, CD3- $\epsilon$ , CD25, or CD69. The extent of TCR downregulation on CD8<sup>+</sup> T cells was normalized using the formula: percentage of TCR downregulation =  $100 - ([\text{TCR mean fluorescence intensity in response to a RMA-S loaded with a given peptide concentration} / \text{TCR mean fluorescence intensity in response to RMA-S loaded with the same concentration of the negative control AV peptide}] \times 100)$ .

## Results

**Generation of Mice with Mutations in Interdomain B of ZAP-70.** Mice with Tyr to Phe mutation at position 292 or 315 of ZAP-70 were generated using the knockin strategy outlined in Fig. 1. As compared with the reconstitution of ZAP-70-deficient mice with transgenes encoding mutant



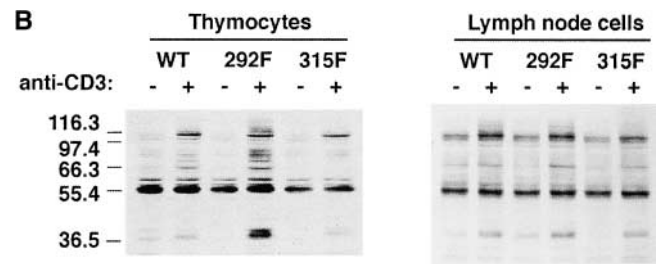
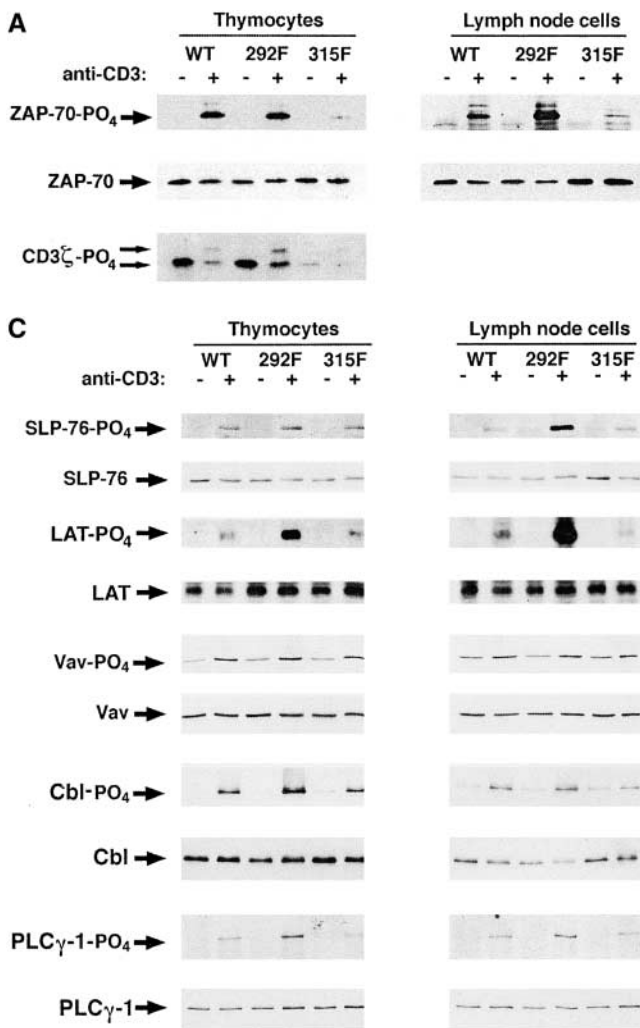
**Figure 1.** Generation and identification of ZAP-70 Y292F and ZAP-70 Y315F mice. ZAP-70 Y292F mice. (A, 1). Partial restriction endonuclease map of the wild-type ZAP-70 gene. Exons 1–7 are shown as black boxes. The restriction sites are: R, Eco RI; and X, XbaI. (A, 2) Targeting vector used for introduction of the ZAP-70 Y292F mutation. The codon present in exon 6 and corresponding to residue Y292 was mutated to phenylalanine. A diagnostic Clal restriction site (C) was simultaneously introduced in the intron 3' of exon 6 to tag the ZAP-70 Y292F mutation. The targeting construct includes a loxP-flanked neo<sup>c</sup> gene in the intron 3' of exon 6. LoxP sites are shown as filled triangles and the neo<sup>c</sup> gene as a dotted box. (A, 3) Structure of the targeted

locus. (A, 4) Final structure of the targeted locus after removal of the neo<sup>c</sup> gene by Cre-mediated recombination. The 5' and 3' probes used to verify the targeting events are indicated as black boxes and the position of the PCR primers (a and b) used to monitor the transmission of the mutant ZAP-70 Y292F allele indicated by triangles. (B) Southern blot analyses of recombinant ES cell clones before deletion of the neo<sup>c</sup> gene. DNA from parental E14 cells and from a clone (292.F5) that gave germline transmission of the modified ZAP-70 allele were digested with XbaI and hybridized with the 3' single-copy probe depicted in A. (C) DNA-PCR analysis of wild-type (w/w), heterozygous (w/m), and homozygous (m/m) littermates using the pair of primers shown in A. The single loxP site remaining in the modified ZAP-70 allele after deletion of the neo<sup>c</sup> gene resulted in an amplified PCR product 140 bp-longer than the one amplified from the wild-type allele. The amplified products were run on an agarose gel and stained with ethidium bromide. ZAP-70 Y315F mice. (D, 1) Partial restriction map of the ZAP-70 gene. The restriction sites are: R, Eco RI; S, Sac I; and X, Xba I. (D, 2) Targeting vector used for introduction of the ZAP-70 Y315F mutation. The codon present in exon 7 and corresponding to residue Y315 was mutated to phenylalanine. A diagnostic Sac I site was simultaneously introduced in exon 7 to tag the ZAP-70 Y315F mutation. The targeting construct includes a loxP-flanked neo<sup>c</sup> gene in the intron 5' to exon 3. (D, 3) Structure of the targeted locus. (D, 4) Structure of the targeted locus after removal of the neo<sup>c</sup> gene by Cre-mediated recombination. The 5' and 3' probes used for Southern blot analyses are shown as black boxes and the position of the PCR primers (c and d) used to monitor the transmission of the ZAP-70 Y315F mutation shown as triangles. (E) Southern blot analysis of recombinant ES clones before deletion of the neo<sup>c</sup> gene. DNA from parental E14 and from clone 315. B6 that gave germline transmission were digested with Xba I and hybridized with the 3' probe shown in D. (F) DNA-PCR analysis of wild-type (w/w), heterozygous (w/m), and homozygous (m/m) littermates using the pair of primers shown in D. The single loxP site left after deletion of the neo<sup>c</sup> gene resulted in an amplified PCR product 140-bp longer than the one amplified from the wild-type allele.

ZAP-70 molecules, the approach we selected readily permits the expression of ZAP-70 mutant molecules at physiological levels and with appropriate kinetics. To induce the deletion of the loxP-flanked neomycin cassette, heterozygous mice carrying either of the mutated ZAP-70 alleles were crossed to the Deleter strain (27). The resulting progeny was backcrossed on a C57BL/6 background and used in further experiments. For the sake of comparison and owing to the high degree of sequence conservation existing between the mouse and human ZAP-70 interdomains B, the tyrosine numbering originally proposed for human ZAP-70 has been conserved for mouse ZAP-70. To confirm that the intended point mutations had been genuinely introduced, ZAP-70 transcripts were cloned by reverse transcription and PCR amplification from the thymus of the mutated mice, and the presence of the Y292F or Y315F mutations confirmed by DNA sequence analysis (data not shown).

Mice deficient in Syk showed no major abnormalities in  $\alpha\beta$  T cell development, whereas in mice deficient in ZAP-70, thymocytes are arrested at the DP stage. Thymocytes lacking both Syk and ZAP-70 are completely blocked at

the transition from the double negative (DN) stage. These genetic studies indicate that Syk and ZAP-70 exert redundant functions only during pre-TCR function, and are consistent with the observation that Syk is quickly down-regulated after the pre-TCR checkpoint (31). Taken together, these data suggest that any phenotypic effect associated with the ZAP-70 Y292F or ZAP-70 Y315F mutation might become manifest only at or following the DP to SP transition. Analysis of the thymus and secondary lymphoid organs of ZAP-70 Y292F and ZAP-70 Y315F mice revealed both a normal cellularity and a normal representation of T lymphocytes expressing surface levels of TCR  $\alpha\beta$ , CD3, CD4, CD8, CD69, CD44, and CD25 comparable to those found in wild-type littermates (data not shown). The absence of abnormal phenotype in the ZAP-70 Y292F and ZAP-70 Y315F mice may result from adaptive mechanisms that have been set in motion to buffer the effect of the ZAP-70 mutations. When placed under the control of the proximal Lck promoter to reach a high level of expression up to the SP stage, a Syk transgene was found capable of restoring the DP to SP transition of ZAP-70-deficient thymocytes (33). Therefore, in the ZAP-70



**Figure 2.** Tyrosine phosphorylation of CD3- $\zeta$ , ZAP-70, Cbl, Vav1, LAT, SLP-76, and PLC $\gamma$ -1 in ZAP-70 Y292F and ZAP-70 Y315F mice. (A) Phosphorylation of ZAP-70 and CD3- $\zeta$  in thymocytes and lymph node T cells in response to TCR cross-linking. Thymocytes (left) or lymph node cells (right) were isolated from wild-type (wt), ZAP-70 Y292F (292F), and ZAP-70 Y315F (315F) mice expressing the P14 TCR transgene. Cells were left unstimulated (-) or stimulated by CD3 cross-linking (+), lysed, and immunoprecipitated with an anti-ZAP-70 antiserum. Samples were then subjected to immunoblotting with an anti-phosphotyrosine antibody (upper and lower panels). The amount of immunoprecipitated ZAP-70 was verified by stripping the membranes and reprobing them with an anti-ZAP-70 monoclonal antibody (middle panels). (B) Effect of ZAP-70 Y292F (292F) and ZAP-70 Y315F (315F) on inducible tyrosine phosphorylation. Thymocytes and lymph node cells from wild-type (wt), 292F and 315F mice expressing the P14 TCR transgene, were isolated and treated as described in A. Postnuclear lysates were separated by SDS-PAGE and analyzed by Western blot analysis with the antiphosphotyrosine antibody 4G10. The molecular weight markers (in thousands) are noted to the left of the blot. (C) Phosphorylation of SLP-76, LAT, Vav1, Cbl, and PLC $\gamma$ -1 in thymocytes and lymph node T cells in response to TCR cross-linking. Cell lysates were immunoprecipitated with antisera specific for the proteins specified in the left margin and each sample analyzed by antiphosphotyrosine immunoblotting. The blots were subsequently stripped and reprobed with the antibodies used in the immunoprecipitation to control for protein level. The molecular species that are detected are noted to the left of the blots.

Y292F and ZAP-70 Y315F thymocytes, a few DP cells adventitiously expressing Syk at high levels may have been specifically selected to give rise to populations of SP cells with unusually high levels of Syk. To compare at the single cell level the levels of Syk in wild-type and ZAP-70 mutant thymi, thymocytes were permeabilized, stained intracellularly with an anti-Syk antibody, and analyzed by flow cytometry (31). This analysis showed that both ZAP-70 mutant thymi contained levels of Syk that were identical to wild-type thymi, and similarly higher in the DN population, decreased in the DP population, and low in SP cells (data not shown). Therefore, in contrast to the compensatory mechanisms that have been previously documented in some Src family kinase deficiencies (34), the pattern of Syk expression during T cell development is similar in wild-type and in the ZAP-70 Y292F and Y315F mutants.

*Phosphorylation of Zap-70 and of CD3- $\zeta$  in Thymocytes and Peripheral T Cells of ZAP-70 Y292F and ZAP-70 Y315F Mice.* We analyzed next the TCR-dependent phosphorylation of ZAP-70 Y292F and ZAP-70 Y315F molecules in both thymocytes and lymph node T cells. As shown in Fig. 2 A, mutation of Y315 markedly impaired the ability of ZAP-70 to become tyrosine phosphorylated after TCR

stimulation, a finding in agreement with previous observations made in DT-40 cells (11). Despite the fact that Y292 constitutes a primary *in vivo/in vitro* tyrosine phosphorylation site (5), ZAP-70 Y292F molecules showed levels of induced tyrosine phosphorylation that were equal (thymocytes) or even greater (lymph node T cells) than wild-type ZAP-70 molecules (Fig. 2 A). The difference noted between thymocytes and lymph node T cells suggests that ZAP-70 tyrosine phosphorylation might be regulated in part via distinct mechanisms in immature and mature T cells. Reprobing the blot with an antibody against ZAP-70 showed that both ZAP-70 mutant proteins and wild-type ZAP-70 were expressed at similar levels (Fig. 2 A, middle panels). Therefore, the differences in inducible tyrosine phosphorylation documented in Fig. 2 A cannot be ascribed to differences in the levels of ZAP-70 expression.

After TCR stimulation, the levels of CD3- $\zeta$  tyrosine phosphorylation were also affected by the presence of the ZAP-70 mutants. Higher amounts of 21- and 23-kD tyrosine phosphorylated forms of CD3- $\zeta$  (p21 and p23) co-precipitated with ZAP-70 Y292F than with wild-type ZAP-70 molecules (Fig. 2 A). Conversely, compared with wild-type ZAP-70 immunoprecipitates, less p21 and p23

CD3- $\zeta$  forms were bound to the ZAP-70 Y315F molecules. This reduction was not due to an indirect effect of the ZAP-70 Y315F mutation on the detergent solubility of the various CD3- $\zeta$  species, as comparable results were obtained when the levels of TCR-inducible CD3- $\zeta$  phosphorylation were assessed by immunoblotting on whole cell lysate or after immunoprecipitation with an anti-CD3- $\zeta$  antiserum (data not shown). It should be noted that the p21 CD3- $\zeta$  species, a phosphoisoform that is constitutively present in wild-type thymocytes and lymph node T cells (35), is also expressed at significantly lower levels in ZAP-70 Y315F thymocytes than in wild-type or ZAP-70 Y292F thymocytes (Fig. 2 A). Considering that ZAP-70 is not responsible for the phosphorylation of the CD3 ITAMs (see Introduction), the reduced amount of phospho- $\zeta$  species unexpectedly observed in resting ZAP-70 Y315F-expressing T cells suggests that the Y315F mutation may have a direct or indirect effect on the ability of the ZAP-70 tandem SH2 domains to bind and protect phosphorylated CD3- $\zeta$  ITAMs (Discussion).

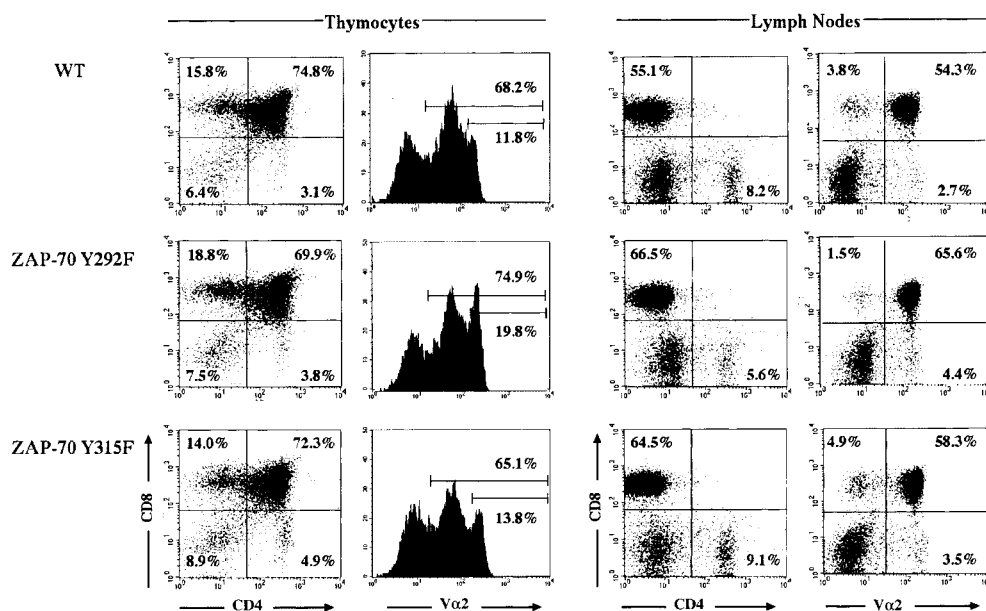
*Phosphorylation of Cbl, Vav1, SLP-76, PLC $\gamma$ -1, and LAT in ZAP-70 Y292F and ZAP-70 Y315F Mutant Mice.* After TCR triggering, the atypical SH2 domain of Cbl binds with high-affinity to ZAP-70 via the phosphotyrosine-containing motif Y<sup>292</sup>TPEP (14, 15), whereas the tyrosine-phosphorylated Y<sup>315</sup> ESP motif found in ZAP-70 has been proposed to be a docking site for the SH2 domain of Vav1 (36, 37). Considering that both Cbl and Vav1 constitute candidate substrates for ZAP-70/Syk PTKs (16, 38), we asked next whether the TCR-induced Cbl and Vav1 tyrosine phosphorylation depends on the presence of the Y292 and Y315 residues, respectively. After TCR cross-linking, ZAP-70 Y292F thymocytes and lymph node T cells exhibited levels of Cbl phosphorylation similar to those found in wild-type cells, whereas the ZAP-70 Y315F-mutant and wild-type T cells present in lymph nodes and thymus displayed comparable levels of phosphorylated Vav1 species (Fig. 2 C). The latter result is in line with previous data, obtained in transformed T cell lines, and showing that the expression of ZAP-70 Y315F mutant molecules did not affect the extent of CD3-induced Vav1 phosphorylation (8, 10). Taken together, our results demonstrate that Y292 and Y315 are dispensable for the TCR-induced tyrosine phosphorylation of Cbl and Vav1, respectively. They further suggest that other molecules present in the TCR signaling cassette (e.g., SLP-76 in the case of Vav1, and Nck or LAT in the case of Cbl; for a review, see reference 39) act as additional adaptors for Cbl and Vav1.

Considering that the TCR-dependent phosphorylation of CD3- $\zeta$  and ZAP-70 is enhanced by the Y292F mutation and attenuated by the Y315F mutation, we also examined whether these differences affect signaling events further downstream in the T cell activation cascade. Probing post-nuclear T cell lysates from wild-type and ZAP-70 mutants with an antiphosphotyrosine antibody showed an increase in the inducible tyrosine phosphorylation of several discrete bands in ZAP-70 Y292F and conversely a reduction in the

antiphosphotyrosine reactivity of the same bands in ZAP-70 Y315F (Fig. 2 B). A more extensive analysis of the phosphorylation state of individual proteins was undertaken. We chose to analyze the phosphorylation levels of the adaptor protein LAT and SLP-76 since they constitutes primary substrates of ZAP-70 (for a review, see reference 39). Compared with wild-type ZAP-70 molecules for their ability to contribute to the TCR-induced phosphorylation of LAT, and SLP-76, ZAP-70 Y315F mutant molecules showed comparable activity when analyzed in thymocytes and similar (SLP-76) or slightly reduced activity (LAT) when analyzed in the context of lymph node T cells (Fig. 2 C). Therefore, the greatly diminished tyrosine phosphorylation of CD3- $\zeta$  and ZAP-70 in ZAP-70 Y315F-expressing T cells does not appear to have a commensurate impact on LAT or SLP-76 phosphorylation. In contrast, when analyzed in lymph node T cells and compared with wild-type ZAP-70, expression of ZAP-70 Y292F molecules markedly increased the TCR-induced tyrosine phosphorylation of LAT and SLP-76 (Fig. 2 C). In thymocytes, the ZAP-70 Y292F mutant significantly increased only the inducible tyrosine phosphorylation of LAT. After anti-CD3 $\epsilon$  antibody stimulation, the wild-type and mutant T cells displayed almost the same degree of tyrosine phosphorylation on phospholipase C (PLC) $\gamma$ -1 (Fig. 2 C). On the basis of these results, we conclude that the ZAP-70 Y292F and ZAP-70 Y315F mutations selectively affected the TCR induced phosphorylation of a subset of signal transducers.

*TCR- $\alpha\beta$  Selection in ZAP-70 Y292F and ZAP-70 Y315F Mutant Thymocytes.* Because of the epigenetic mechanisms that shape the repertoire of TCRs expressed on mature T cells, selection of TCRs with lower or higher affinity for self-MHC might have compensated for the ZAP-70 Y292F and ZAP-70 Y315F mutations, respectively. When tested in mice in which TCR variability is neutralized by coexpressing a transgenic TCR originally calibrated in the context of a normal TCR signaling cassette, the effects of the ZAP-70 Y292F or ZAP-70 Y315F mutations may become more salient. Therefore, each ZAP-70 mutation was introduced into two different TCR transgenic lines. One, denoted H-Y, expresses a TCR specific for the male H-Y antigen (24, 25), and the other one, denoted P14, a TCR specific for a peptide derived from the lymphocytic choriomeningitis virus glycoprotein (23). In both instances, the cognate peptide is presented in the context of H-2D<sup>b</sup> molecules.

As shown in Fig. 3, the analysis of ZAP-70 mutant mice carrying the P14 TCR transgene did not reveal any major effect of either mutation on the generation of CD8<sup>+</sup>/V $\alpha$ 2<sup>high</sup> thymocytes and lymph node T cells. When compared with the P14 TCR, the H-Y TCR appears to bind with a lower affinity to its selecting ligand (40), and thus might constitute a more sensitive reporter of the signaling capacity of the ZAP-70 Y292F and Y315F molecules. In wild-type female thymi, the interaction of the H-Y TCR with unknown self ligand(s) results in the selection of CD8<sup>+</sup> SP cells that express high levels of the transgenic TCR (detected by the specific antibody T3.70). As shown



**Figure 3.** T cell development in ZAP-70 Y292F and ZAP-70 Y315F mice expressing the P14 transgenic TCR. Thymocytes and total lymph node cells from wild-type (WT), ZAP-70 Y292F, and ZAP-70 Y315F mice expressing the P14 TCR transgene were analyzed by three-color flow cytometry for expression of CD4, CD8, and V $\alpha$ 2. For two-color plots, the percentage of cells found in each quadrant is indicated. For single-color profiles of total thymocytes the filled histograms depict the levels of V $\alpha$ 2 staining observed on total thymocytes. Also shown is the percentage of V $\alpha$ 2-positive cells scoring within either the TCR intermediate plus high windows or in the TCR high window alone. The slight increase in the percentage of V $\alpha$ 2<sup>high</sup> cells noted in ZAP-70 Y292F thymocytes was not found significant when data from a larger number of mutant mice were analyzed.

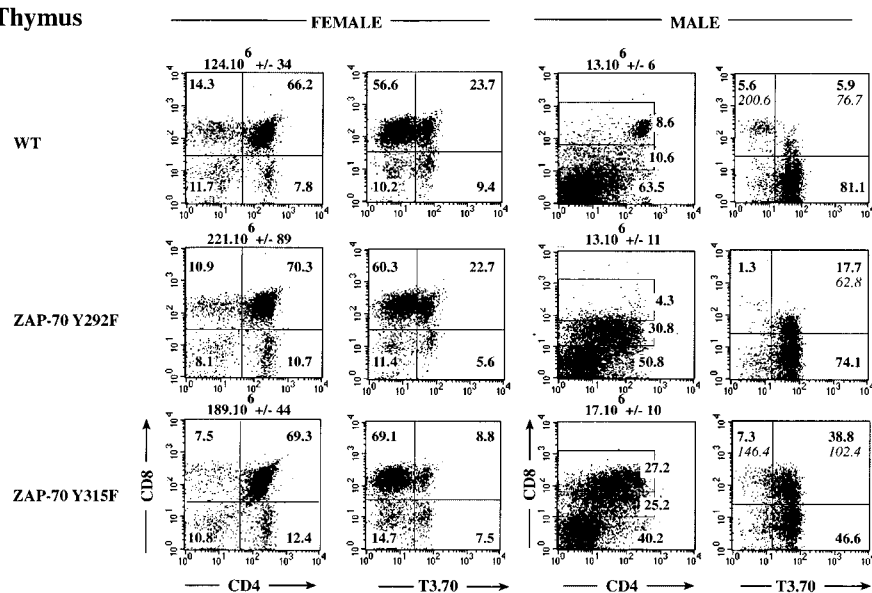
in Fig. 4, the Y292F mutation did not detectably affect positive selection as documented by the number and percentage of CD8<sup>+</sup>/T3.70<sup>high</sup> cells found in the thymus and the lymph nodes. This suggests either that the Y292F mutation has no enhancing effect on positive selection, or that in the H-Y TCR system, this developmental transition is already operating at a maximum rate. In contrast, the percentage and total number of CD8<sup>+</sup>/T3.70<sup>high</sup> cells found in the thymus and the lymph nodes of ZAP-70 Y315F mice showed a consistent reduction (two to threefold range) when compared with wild-type and ZAP-Y292F mice (Fig. 4).

Due to a premature expression of the H-Y TCR transgene, the T cells that arise in H-Y TCR transgenic male thymus are negatively selected before or at the transition to the DP stage (41). This results in the depletion of the DP and SP populations and in a severely reduced thymic cellularity. This last feature was unchanged after introducing the H-Y TCR on a ZAP-70 Y292F or ZAP-70 Y315F background (Fig. 4). However, when compared with H-Y TCR wild-type thymi, H-Y TCR/ZAP-70 Y315F thymi showed a novel T3.70<sup>high</sup>/DP cell population that represented ~30% of the thymocytes. Its appearance does not result in a concomitant increase in thymic cellularity, or in the presence of peripheral CD8<sup>+</sup>/T3.70<sup>high</sup> cells. Therefore, negative selection is still taking place in H-Y TCR/ZAP-70 Y315F mice. However, due to a probable decrease in the TCR signaling potential of the ZAP-70 Y315F-expressing T cells, negative selection is in part delayed until the DP stage of T cell development. Conversely, the enhanced TCR signaling potential thought to be associated with the presence of ZAP-70 Y292F molecules (see above), should have resulted in a more effective

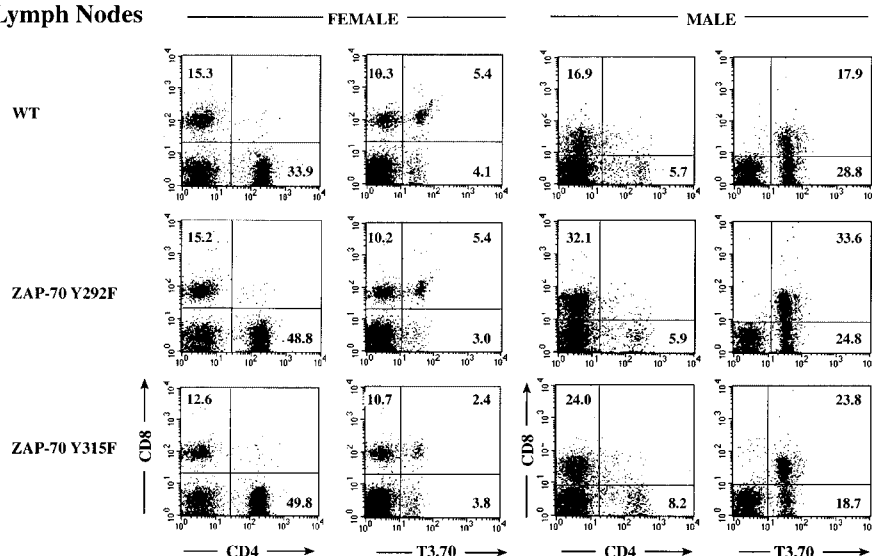
negative selection. Consistent with this view, the expression of ZAP-70 Y292F prevented the development of T3.70<sup>-</sup>/DP cells (Fig. 4). These cells normally arise in H-Y TCR transgenic male thymus after the expression of an endogenous TCR  $\alpha$  chain capable of displacing the transgenic TCR  $\alpha$  chain from the cell surface, and therefore of protecting them from negative selection. By exacerbating the efficiency of negative selection at the DN stage, it is likely that the ZAP-70 Y292F mutation prevented the occurrence of endogenous TCR  $\alpha$  gene rearrangements and thus totally blocked the emergence of T3.70<sup>-</sup>/DP cells.

CD5, a cell surface molecule expressed on thymocytes and on most mature T cells, functions as a negative regulator of TCR-mediated signaling (42). Its expression is up-regulated on DN cells via signaling by the pre-TCR. On DP cells, intermediate CD5 levels are maintained by low affinity TCR-MHC interactions (43, 44). Wild-type, ZAP-70 Y292F, and ZAP-70 Y315F DN and DP cells isolated from P14 or female H-Y TCR transgenic thymi expressed similar levels of CD5 (Fig. 5, and data not shown), an observation consistent with the view that any effect associated with the ZAP-70 mutations should be detectable only during or after TCR  $\alpha\beta$  selection (see above). Interestingly, the levels of CD5 expression reached on SP cells have been shown to be proportional to the TCR signaling capacity (43). For instance, expression of an  $\alpha\beta$  TCR with a lowered signaling potential is generally accompanied by the selection of SP cells expressing lower levels of the CD5 negative regulator (43). In this context, SP thymocytes from both H-Y TCR/ZAP-70 Y315F and P14 TCR/ZAP-70 Y315F mice were found to express lower surface levels of CD5 than SP thymocytes from the same TCR transgenics bred on a wild-type or a ZAP-70 Y292F back-

## Thymus



## Lymph Nodes



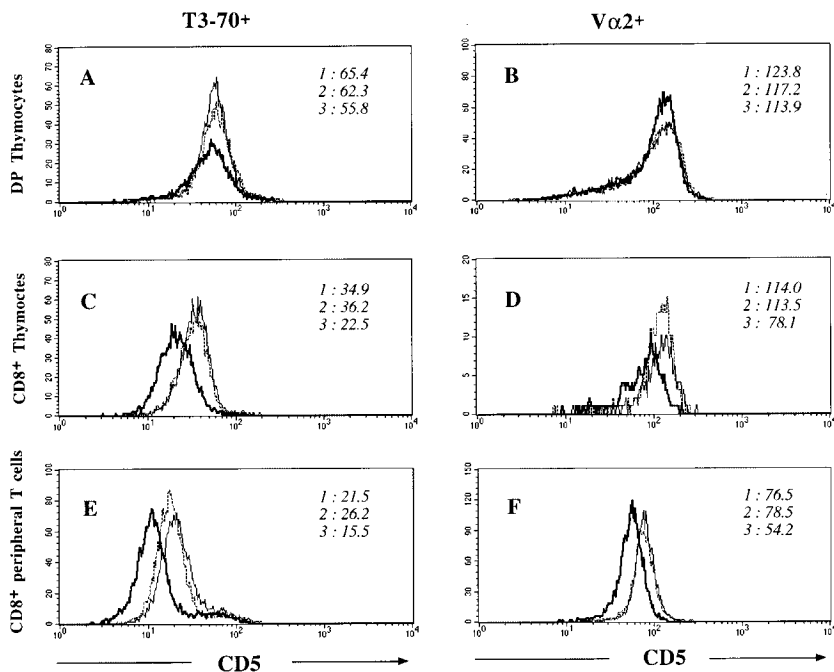
**Figure 4.** T cell development in ZAP-70 Y292F and ZAP-70 Y315F mice expressing the H-Y transgenic TCR. Thymocytes and total lymph node cells from wild-type (WT), ZAP-70 Y292F, and ZAP-70 Y315F female or male mice expressing the H-Y TCR transgene were analyzed by three-color flow cytometry for expression of CD4, CD8, and of the transgenic TCR  $\alpha$  chain used by the H-Y TCR (T3.70). The percentage of cells found in each window is indicated. In the two-color plots shown in the right-hand part of the upper set, the values in italic correspond to the mean CD8 fluorescence intensity found on T3.70<sup>+</sup>/CD8<sup>+</sup> thymocytes. Numbers above the plots indicate the total number of thymocytes  $\pm$  SD. These data are representative of six experiments.

ground (Fig. 5). Moreover, this relative difference in CD5 surface expression was maintained on lymph node T cells (Fig. 5). Therefore, this observation further suggests that TCRs functioning in the context of ZAP-70 Y315F mutant molecules have a lowered signaling potential, and that the corresponding T cells rely on CD5-based compensatory adjustments to reach the signaling threshold required for positive selection, in that only those cells with a sufficiently low level of CD5 expression could be positively selected.

**Effects of the ZAP-70 Mutations on TCR/CD3 Downregulation and Expression of Surface Activation Markers.** The observation that both engineered ZAP-70 mutations do not detectably affect the development of P14 TCR transgenic cells offers the possibility to address *ex vivo* the functional properties of mature T cells expressing ZAP-70 Y292F or ZAP-70 Y315F mutant kinases. If we except the difference previously noted for CD5, all the CD8<sup>+</sup> cells found

in the periphery of ZAP-70 mutant mice express the same surface phenotype and qualify as naive resting T cells, in that they express a CD25<sup>-</sup> CD44<sup>low</sup>, CD62L<sup>high</sup>, and CD69<sup>-</sup> phenotype (data not shown). CD8<sup>+</sup> lymph node T cells were purified from wild-type, ZAP-70 Y292F, and ZAP-70 Y315F mice expressing the P14 TCR transgene and cultured with RMA-S cells pulsed with graded doses of peptides p33 or AV. p33 constitutes a strong agonist for the P14 TCR (45), whereas AV, an adenovirus peptide known to efficiently bind to H-2D<sup>b</sup>, was used as a negative control (46). After 15 h of culture, cells were harvested and stained for V $\alpha$ 2, CD3 $\epsilon$ , CD25, or CD69 expression. Fig. 6 shows the expression of these various markers on gated CD8<sup>+</sup> T cells. Upon challenge with the agonist peptide, T cells from ZAP-70 Y292F and ZAP-70 Y315 mice were as efficient as their wild-type counterpart at downregulating their TCR/CD3 complexes and at up-



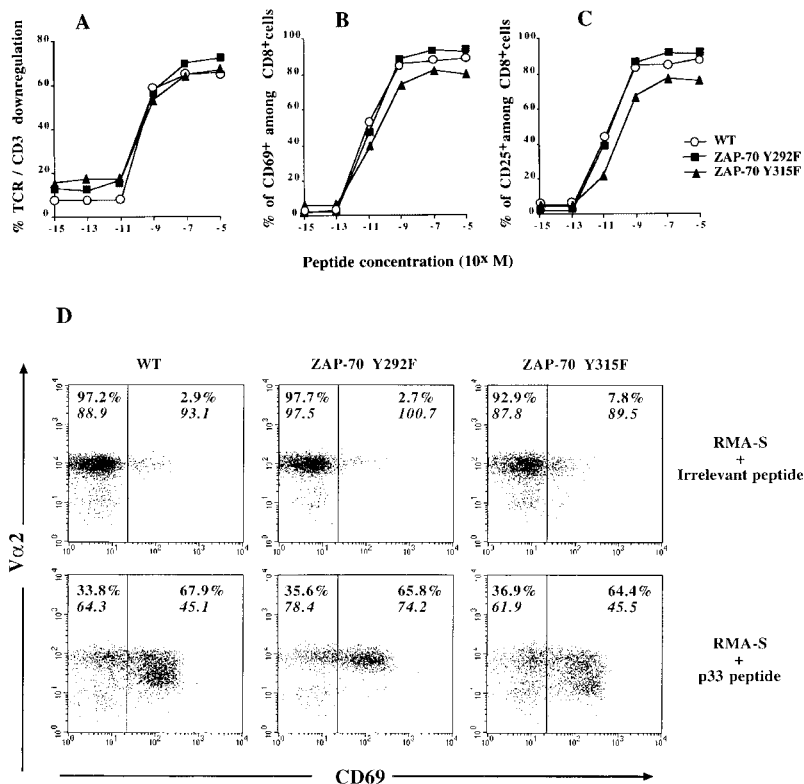


**Figure 5.** CD5 expression on DP thymocytes, SP CD8<sup>+</sup> thymocytes, and CD8<sup>+</sup> peripheral T cells from P14 and female H-Y transgenic mice expressing mutated ZAP-70 molecules. Comparison of CD5 levels on DP thymocytes (A and B), CD8<sup>+</sup> SP thymocytes (C and D), and CD8<sup>+</sup> lymph node cells (E and F) from H-Y and P14 transgenic mice expressing wild-type (dotted line), ZAP-70 Y292F (thin line), or ZAP-70 Y315F (bold line) molecules. Cells were analyzed by four-color cytometry for expression of CD4, CD8, CD5, and T3.70 (H-Y mice) or Vα2 (P14 mice). Single-color histograms represent CD5 staining on transgenic, clonotype positive cells. Numbers at the top right part of each panel indicate the mean CD5 fluorescence intensity found on clonotype-positive cells expressing wild-type (1), ZAP-70 Y292F (2), or ZAP-70 Y315F (3) molecules. These data are representative of four experiments.

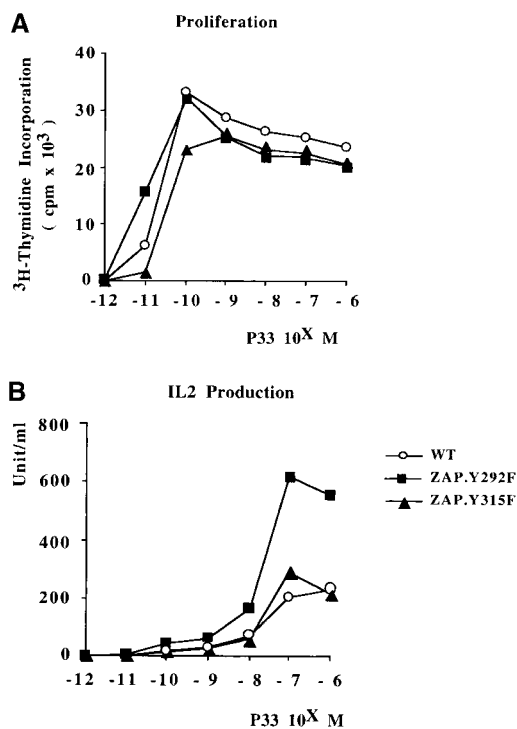
regulating the CD25 and CD69 molecules. When TCR expression was measured at earlier time points, the degree of TCR downmodulation was lower on ZAP-70 Y292F T cells, suggesting a slower rate of antigen-induced TCR internalization on these cells as compared with wild-type and ZAP-70 Y315F T cells (Fig. 6 D). No TCR/CD3

downregulation and no CD25 or CD69 upregulation were seen on Vα2<sup>-</sup>/CD4<sup>+</sup> T cells or with the negative control peptide AV (data not shown).

*Effects of the ZAP-70 Mutation on Proliferation and Cytokine Production.* When challenged with the p33 peptide, CD8<sup>+</sup> lymph node T cells purified from ZAP-70 mutant



**Figure 6.** TCR downregulation and expression of CD25 and CD69 on CD8<sup>+</sup> T cells isolated from P14 mice expressing wild-type ZAP-70 (wt), ZAP-70 Y292F, and ZAP-70 Y315F molecules and after stimulation with the p33 agonist peptide. RMA-S cells ( $2.5 \times 10^5$ ) were pre-pulsed with graded concentrations of antigenic peptide and cultured with T cells ( $1.5 \times 10^6$ ) purified from P14 transgenic mice expressing wild-type or mutant ZAP-70 molecules. After 12–15 h, levels of TCR/CD3 complexes, CD25 and CD69 were determined by flow cytometry on gated CD8<sup>+</sup> T cells. One representative experiment of two is shown. (A) TCR downregulation. Data are given as percent mean fluorescence intensity of T cells cultivated for 12–15 h with RMA-S cells pulsed with an irrelevant H-2 D<sup>b</sup>-binding peptide. Similar values were obtained by using either anti-Vα2 or anti-CD3-ε antibodies. (B and C) CD69 and CD25 upregulation. Data show the percent of CD69<sup>+</sup> (B) or CD25<sup>+</sup> (C) cells found among CD8<sup>+</sup> T cells. (D) Downmodulation of surface TCR and upregulation of CD69 on lymph node T cells isolated from the specified mice after 2.5 h of stimulation with RMA-S cells that were pre-pulsed with an irrelevant peptide (top), or with the p33 peptide (bottom). Gated CD8<sup>+</sup> cells were analyzed for expression of Vα2 and CD69 after 2.5 h of culture. Numbers in each panel indicate the percentage of cells and their TCR mean fluorescence intensity value. These data are representative of four experiments.



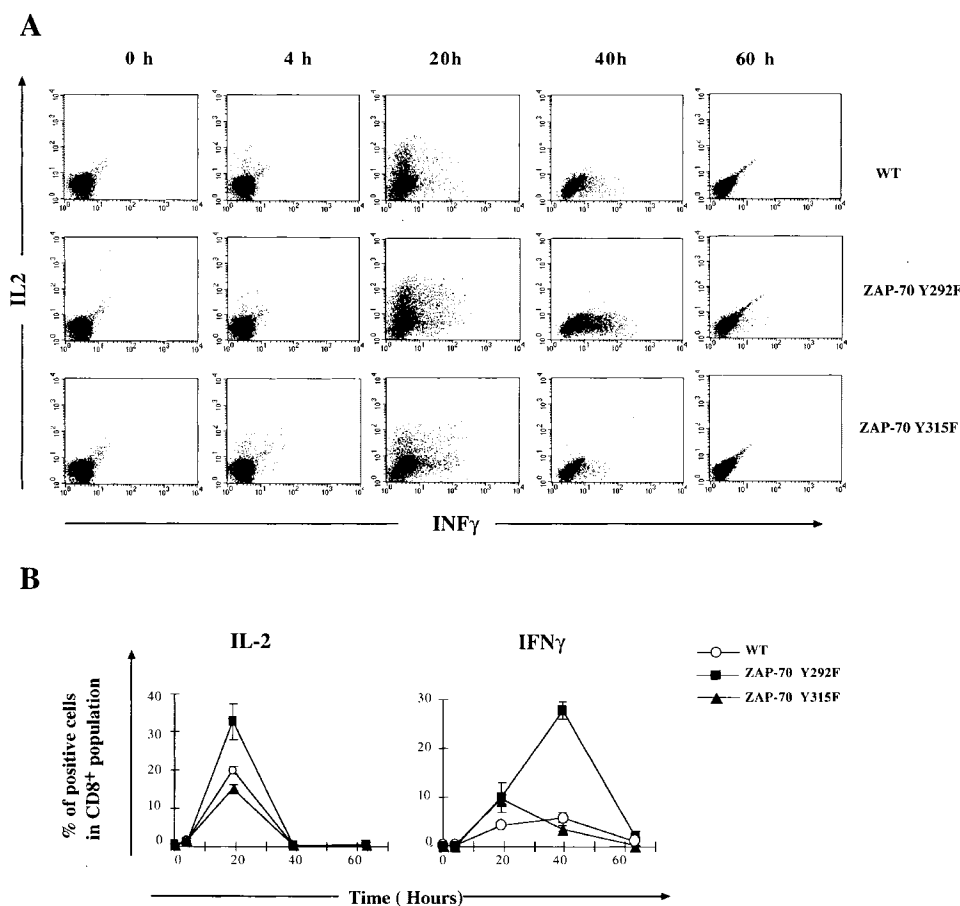
**Figure 7.** Proliferative responses and IL-2 secretion. H-2 D<sup>b</sup>-positive spleen cells ( $5 \times 10^5$ ) isolated from CD3 $\epsilon$ -deficient mice were pulsed for 2 h with graded concentrations of p33 agonist peptide and subsequently cultured with purified CD8<sup>+</sup> T cells isolated from P14 mice expressing wild-type ZAP-70 (wt), ZAP-70 Y292F, or ZAP-70 Y315F molecules. (A) After 40 h, T cell proliferation was assessed by pulsing cultures with [<sup>3</sup>H]TdR for 8 h. (B) After 40 h, supernatants were assayed for IL-2 contents. Data correspond to the mean response of independent cultures established from two mice of each genotype. One representative experiment of three is shown.

mice expressing the P14 TCR transgene showed proliferative dose-response curves almost superimposable to those obtained with wild-type P14 T cells (Fig. 7). However, when challenged with a subnanomolar ( $10^{-11}$  M) concentration of p33 peptide, ZAP-70 Y292F-expressing cells consistently proliferated more than wild-type P14 T cells, whereas no proliferation was observed for ZAP-70 Y315F-expressing cells. To follow the kinetics of division of individual P14<sup>+</sup> T cells expressing wild-type or mutant ZAP-70 molecules in response to suboptimal concentrations ( $10^{-8}$  to  $10^{-9}$  M) of p33 agonist peptide, we used the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). CFSE segregates equally between daughter cells upon cell division, resulting in the sequential halving of cellular fluorescence intensity within each successive generation (47). Analysis of the flow cytometry profiles measured at time points extending up to 72 h, and calculation of the corresponding division index (48) did not reveal any differences between P14<sup>+</sup> T cells expressing wild-type and ZAP-70 mutant molecules. When lymph node T cells from ZAP-Y292F/P14, ZAP-Y315F/P14, and wild-type P14 mice were stimulated in vitro with suboptimal dose of p33 agonist peptide or of the partial agonist peptides M3V and

L3V, the cytotoxic effectors generated after a 3 d-culture were all capable of efficiently lysing RMA-S target cells pulsed with suboptimal concentration of p33 ( $10^{-9}$  M), M3V ( $10^{-7}$  M), or L3V ( $10^{-6}$  M) peptides (data not shown). During these comparative analyses, the only robust difference was found at the level of IL-2 production. Whereas P14 T cells expressing ZAP-70 wild-type and ZAP-70 Y315F molecules produced equivalent levels of IL-2 in response to graded concentrations of p33 peptide, P14 T cells expressing ZAP-70 Y292F molecules consistently yielded two to threefold more IL-2 (Fig. 7 B). This enhanced IL-2 production may result from a greater response per cell or from a greater number of responding cells, with a production per cell remaining relatively constant. To settle this issue, we analyzed cytokine production at the single cell level, using a flow cytometric assay for intracellular cytokine levels. IL-2 and IFN- $\gamma$  production tests were selected since P14<sup>+</sup> T cells stimulated with suboptimal dose of agonist peptide and without addition of exogenous cytokines are capable of producing readily detectable levels of these two cytokines (49). As shown in Fig. 8 A, naive CD8<sup>+</sup> T cells purified from mice expressing wild-type and mutant ZAP-70 molecules expressed neither IL-2 nor IFN- $\gamma$ . After in vitro stimulation with the p33 peptide, the number of IL-2-producing T cells reached a maximum within 20 h and rapidly declined to baseline levels at 40 h, whereas the number of IFN- $\gamma$ -producing T cells found in each culture reached a plateau between 20 and 40 h of culture, and declined thereafter. At 20 h, the majority of responding cells make either IFN- $\gamma$  or IL-2, and only a minority of them produced both cytokines. Therefore, expression of ZAP-70 mutants molecules had no marked effect on the kinetics of cytokine production, and resulted in a rather normal range of IL-2 and IFN- $\gamma$  staining intensities. However, as shown on Fig. 8 B, in the presence of the ZAP-70 Y292F molecules, the percentages of IL-2- or IFN- $\gamma$ -producing cells were consistently greater than those reached in cell populations expressing ZAP-70 wild-type or ZAP-70 Y315F molecules. Therefore, this difference is consistent with that already documented when IL-2 production was measured at the cell population level (Fig. 7 B). Importantly, these results indicate that the enhanced TCR signaling potential resulting from the ZAP-70 Y292F mutation does not lead to a greater range of IL-2 (or IFN- $\gamma$ ) production per cell, but rather translates into a greater number of responding T cells. They also demonstrate that the minute amounts of ZAP-70 phosphorylation that are induced by TCR stimulation in ZAP-70 Y315F T cells (Fig. 2 A) are nonetheless sufficient to activate the pathways leading to cytokine production and cell cycle entry.

## Discussion

The present in vivo and ex vivo analyses extend previous studies based on transformed cell lines, and show that Y292 plays a role in the attenuation of TCR signaling, whereas Y315 enhances ZAP-70 function. The regulatory proper-



**Figure 8.** Cytokine production analyzed at the single cell level.  $CD8^+$  cells purified from P14 mice expressing wild-type (wt), ZAP-70 Y292F, or ZAP-70 Y315F molecules were cultured in the presence of  $CD3\epsilon$ -deficient spleen cells and of suboptimal concentration ( $10^{-8}$  M) of the p33 agonist peptide. At the specified time, both  $IFN-\gamma$  and IL-2 levels were quantified by intracellular staining. (A)  $IFN-\gamma$  versus IL-2 two-color plots after gating on  $CD8^+$  cells. Before intracellular staining cells were incubated for 4 h in the presence of monensin. (B) The percentage of  $CD8^+$  T cells expressing  $IFN-\gamma$  or IL-2 is shown for various culture time points. Data correspond to the mean response ( $\pm$  SD) of independent cultures established from two mice of each genotype. One representative experiment of four is shown.

ties of phospho-Tyr 315 have been attributed to its ability to recruit Vav1 (11). In Vav1-deficient mice, both the DN to DP and DP to SP transitions are partially blocked, and the total number of mature  $CD4^+$  and  $CD8^+$  T cells reduced twofold when compared with wild-type mice. Analysis of Vav1-deficient T cells, further showed that they express normal levels of TCR and CD5, and that after TCR stimulation, Vav1 has an important role in cell cycle progression and IL-2 production (50). We showed that when analyzed in the context of the “sensitized” genetic background provided by the H-Y TCR transgenic line, the ZAP-70 Y315 mutation reduced the rate of positive selection and delayed the occurrence of negative selection up to the DP stage. However, P14<sup>+</sup> lymph node T cells expressing ZAP-70 Y315F were as efficient as wild-type P14<sup>+</sup> T cells when tested for their ability to proliferate and secrete  $IFN-\gamma$  and IL-2. Although, the decreased levels of CD5 found on ZAP-70 Y315F mature T cells precludes a fair comparison of their properties with those found in Vav1-deficient mice, the effects of the ZAP-70 Y315F mutation appear however milder than those resulting from the lack of Vav1. Along this line, it should be noted that in marked contrast to Vav1<sup>-/-</sup> thymocytes (51), ZAP-70 Y315F thymocytes display TCR-induced  $Ca^{2+}$  responses similar to those found in wild-type thymocytes (data not shown). That Vav1 ablation and Y315F are not equivalent is also indicated by the fact that some very early events induced

by TCR engagement, in particular the tyrosine phosphorylation of the  $CD3-\zeta$  chain, are unaffected by the absence of Vav1 but clearly defective in ZAP-70 Y315F-expressing T cells (see below).

The asymmetrical contributions of residue Y315 of ZAP-70 and of Vav1 to both T cell development and function suggest that among the multiple phosphotyrosine-based motifs that connect Vav1 to the TCR transduction cassette (for a review, see reference 39), the one found in ZAP-70 might not play a major role. Consistent with the existence of additional Vav1-entry sites, ZAP-70 Y315F-expressing T cells retained the ability to phosphorylate Vav1 upon TCR triggering. Whether the ablation of the Vav1 docking site(s) found in SLP-76 more appropriately recapitulates the phenotype observed in Vav1-deficient mice remains to be tested. Considering that the existence of a direct interaction between the phosphorylated-Y<sup>315</sup>ESP motif and Vav1 has not yet been validated on a genetic basis (i.e., by establishment of interaction suppressors or enhancers), it remains formally possible that the effects noted in ZAP-70 Y315F mice do not directly result from a lack of interaction between Vav1 and ZAP-70. For instance, the ZAP-70 Y315F mutation reduced both the constitutive phosphorylation of  $CD3-\zeta$  and the inducible phosphorylation of  $CD3-\zeta$  and ZAP-70 (Fig. 2 A), two features that are notably absent in Vav1-deficiency (52, 53). When tested in Syk-deficient DT-40 chicken cells, mutation of residue

Y315 similarly reduced the antigen receptor-inducible ZAP-70 tyrosine phosphorylation, without exerting any detectable effect on the kinase activity of ZAP-70 and on its capacity to bind to phosphorylated ITAMs (11). We recently confirmed that mutation of residue Y315 does not affect the intrinsic kinase activity of ZAP-70. However, in contrast to the results obtained in DT-40 cells, using cell lysates prepared from thymocytes and lymph node T cells, we found that the ZAP-70 Y315F molecules bind to bi-phosphorylated ITAMs with an affinity that is two to threefold lower than that of wild-type ZAP-70 molecules (data not shown). This difference in affinity may suffice to reduce the ability of the tandem SH2 domains to protect phosphorylated ITAMs from the constitutive action of tyrosine phosphatases, and may account for the markedly reduced levels of the CD3- $\zeta$  p21 and p23 phosphoisoforms, and for the reduced phosphorylation of ZAP-70 and LAT found in ZAP-70 Y315-expressing T cells. Importantly, a Tyr to Phe substitution at either position 292 or 319 of interdomain B did not produce a similar destabilizing effect (Fig. 2 A, and data not shown). Therefore, once phosphorylated, Y315 may specifically contribute, in a direct or indirect mode, to enhance the binding of ZAP-70 to phosphorylated ITAMs. Consistent with this possibility, comparison of Syk and of SykB, a naturally occurring isoform of Syk that lacks 23 amino acids in interdomain B, already showed that interdomain B can regulate the ability of Syk to bind ITAMs (54). Regardless of the exact mechanism through which Y315 modulates the binding ability of the ZAP-70 SH2 domains, our data suggest that interdomain B of ZAP-70 also takes part in the control of the dynamic interactions that exist between ITAMs and ZAP-70 (20, 55, 56). Finally, the observation that the defective phosphorylation of ITAMs and ZAP-70 in Y315F mutant mice correlates with a decreased phosphorylation of LAT but not Vav1 (Fig. 2), further suggests that the former but not the latter is a direct substrate of ZAP-70.

A wealth of biochemical and structural data suggests that the negative regulatory properties of residue Y292 results from its ability to recruit the Cbl and Cbl-b adaptors (14, 57, 58). Cbl and Cbl-b share a high degree of homology over their NH<sub>2</sub>-terminus SH2 and RING finger domains, but diverge almost immediately after the RING finger domain. The RING finger found in Cbl can recruit E2 ubiquitin-conjugating enzymes and function as an E3 ubiquitin ligase (for a review, see reference 15). Compared with wild-type, in both Cbl-deficient and ZAP-70 Y292F thymocytes, CD3- $\zeta$ , ZAP-70, and LAT molecules become more tyrosine phosphorylated after TCR cross-linking (59–61). In contrast, the loss of Cbl-b has no notable effect on the inducible tyrosine phosphorylation of CD3- $\zeta$ , ZAP-70 and LAT, and Cbl-b<sup>-/-</sup> T cells display a distinctive hyperproduction of IL-2 but not of IFN- $\gamma$  that distinguish them from ZAP-70 Y292F T cells (57, 62). Therefore, the partially overlapping phenotype found in thymocytes and T cells from Cbl-deficient and ZAP-70 Y292F mice is consistent with the assumption that ZAP-70 and Cbl interact via the Tyr 292-containing motif. After TCR cross-

linking, ZAP-70 Y292F-expressing T cells retained, however, their ability to increase the tyrosine phosphorylation of Cbl. Although these results indicate that multiple Cbl docking sites exist within the TCR signaling cassette, it should be emphasized that each of them may not be functionally equivalent. For instance, the rigid arrangement existing between the SH2 and RING E3 domains of Cbl (15) may result in a highly selective ubiquitin transfer, and a distinct spectrum of lysine residues is likely to be targeted according to the docking site used by the Cbl SH2 domain. The exact biochemical mechanisms by which the ZAP-70 Y292F mutation enhances TCR signaling remain to be determined. Once bound to the Y292-based motif, Cbl may prevent the docking of a positive regulator to ZAP-70 (for a review, see reference 63). Alternatively, by being brought in contiguity with the proximal components of the TCR transduction cassette, the COOH terminus of Cbl may act as a scaffold to which negative regulators bind. However, based both on the presence of a RING E3 domain adjacent to the Cbl SH2 domain, and on the fact that the CD3 subunits undergo activation-dependent multiubiquitination (64, 65), it is tempting to speculate that the Y292/Cbl interaction enhances the ubiquitin-dependent downregulation of the activated TCR, as previously documented for receptors for the epidermal growth factor and the colony-stimulating factor 1 (for a review, see reference 66). Consistent with this hypothesis, Cbl deficiency resulted in slightly higher steady state levels of TCR-CD3 complexes (58, 59). Although we have not been able to detect a similar increase in the amount of TCR expressed at the cell surface, we observed a delayed downmodulation of the TCR after antigen stimulation (Fig. 6 D). This last observation is consistent with the fact that the internalization motifs present in CD3- $\gamma$  and CD3- $\delta$  (67) play no major role in antigen-induced TCR downregulation (68). However, it remains to establish whether the slower antigen-induced TCR downregulation rate noted in ZAP-70 Y292F-expressing T cells directly results from attenuated TCR ubiquitination, and permits a larger fraction of T cells to reach the elicitation threshold required for the production of IL-2 and IFN- $\gamma$  (Figs. 6 and 8). Finally, it is interesting to note that both the ZAP-70 mutations we have engineered and the deficiencies in Cbl or Cbl-b modify rather than ablate the transducing cassette operated by the TCR. In *Caenorhabditis elegans*, mutations of the Cbl homologue Sli-1 alone does not produce any observable phenotypic changes unless bred on sensitized genetic backgrounds expressing weakly active alleles of an epidermal growth factor receptor homologue (69). Similarly, when introduced into a sensitized Vav1-deficient background, the Cbl-b null mutation was found capable of relieving the signaling defects found in Vav1<sup>-/-</sup> T cells (70). Based on the latter example, it is expected that further crossing of the ZAP-70 Y292F and Y315F mutations onto mouse strains expressing weak alleles of the various components of the TCR transduction cassette will exacerbate their modifier properties and thus contribute to pinpoint the mechanisms by which these two residues exert their regulatory effects.

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