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Negative regulation of TCR signaling by ubiquitination of Zap-70 Lys-217

Elitza Ivanova¹ and Nick Carpino^{1,*}

¹Department of Molecular Genetics and Microbiology, Room 130, Life Sciences Building, Stony Brook University, Stony Brook, NY 11794-5222

Abstract

The tyrosine kinase Zap-70 is a key regulator of T cell receptor (TCR) signaling downstream of antigen presentation, with coordinated regulation of Zap-70 kinase activity critical for proper T cell proliferation, differentiation, and effector function during an immune response. Zap-70 is cytosolic in unstimulated T cells, but is rapidly recruited to the TCR complex following receptor stimulation. Its activity is regulated both by binding to subunits of the TCR and by phosphorylation on multiple tyrosine residues. Zap-70 also has been reported to be ubiquitinated following TCR stimulation. Herein, we confirm the ubiquitination of Zap-70 in T cell lines and in primary human and mouse T cells, and report the identification of nine novel Zap-70 ubiquitination sites. Three sites, including Lys-193, Lys-217, and Lys-376, displayed greater than 20-fold increase in modification levels following TCR stimulation. Abrogation of Lys-217 ubiquitination results in increased kinase activation, enhanced activation of downstream signaling pathways, and elevated IL-2 production following TCR stimulation. These data suggest that Zap-70 ubiquitination contributes to the regulation of Zap-70 signaling following TCR stimulation.

Keywords

Zap-70; ubiquitination; TCR signaling

1. Introduction

The multi-subunit T cell antigen receptor (TCR) plays a central role in the development and function of T lymphocytes. The TCR has no intrinsic catalytic activity and depends on the recruitment and activation of protein tyrosine kinases for transmission of an activation signal (Smith-Garvin et al., 2009). Engagement of the TCR by an MHC receptor – foreign peptide antigen complex leads to Lck-dependent phosphorylation of tyrosine residues within the Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) within different TCR subunits

*To whom correspondence should be addressed. Tel: (631)-632-4610; Fax: (631)-632-9797; nicholas.carpino@stonybrook.edu.

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(Love and Hayes, 2010). This allows recruitment and activation of the Syk family kinase Zap-70 to the TCR complex, where it is phosphorylated and activated (Latour and Veillette, 2001; Smith-Garvin et al., 2009). Zap-70 plays a critical role in propagating signals initiated by TCR engagement and is required for activating many signaling pathways downstream of the activated TCR. The importance of Zap-70 in TCR signaling is underscored by the observation that loss of expression or function of Zap-70 leads to severe developmental and functional defects within the T cell compartment (Au-Yeung et al., 2009; Fischer et al., 2010; Wang et al., 2010). Zap-70 contains two N-terminal SH2 domains that are separated by a linker region termed Interdomain A, and a C-terminal kinase domain. The region between the second SH2 domain and the kinase domain, termed Interdomain B, contains three tyrosine residues (Tyr-292, Tyr-315, and Tyr-319) that are critical for Zap-70 regulation (Au-Yeung et al., 2009).

Zap-70 activation is a multi-step process and is regulated both by binding to phosphorylated ITAMs and by phosphorylation of multiple residues in Zap-70 itself (Au-Yeung et al., 2009; Fischer et al., 2010; Wang et al., 2010). Recruitment of Zap-70 into the TCR complex destabilizes its cytosolic autoinhibited conformation and exposes tyrosines 315 and 319, whose phosphorylation by Lck further destabilizes Zap-70 autoinhibitory interactions. Phosphorylation of Tyr-493 within the Zap-70 kinase domain results in full kinase activation (Au-Yeung et al., 2009; Fischer et al., 2010; Wang et al., 2010), leading to activation of downstream signaling pathways that are important for T cell proliferation, differentiation, and effector functions (Chakraborty and Weiss, 2014; Holloway et al., 2002; Huang and Wange, 2004).

In order to prevent excessive and deleterious lymphocyte responses, T cells possess a number of mechanisms to limit the intensity and duration of signals generated by receptor activation (Acuto et al., 2008). Following antigen stimulation, the TCR is rapidly internalized to limit the pool of surface receptor available for stimulation (von Essen et al., 2004). In addition, dephosphorylation of kinase activating residues and phosphorylation of kinase inhibitory residues serve to regulate signal initiation and propagation (Pao et al., 2007). Ubiquitin ligases are also believed to target components of the TCR signaling complex for internalization and/or degradation (Liu, 2004). Another mechanism for negatively regulating signals downstream of the TCR involves the Suppressor of T cell receptor Signaling (Sts) proteins, Sts-1 and Sts-2. The Sts proteins are two related phosphatases that are characterized by a unique tripartite structure comprised of an N-terminal ubiquitin-association domain (UBA), a central SH3 domain, and a C-terminal histidine phosphatase (HP) domain. Zap-70 is negatively regulated by the Sts phosphatases, as demonstrated by its hyper-phosphorylation in stimulated *Sts-1/2^{-/-}* T cells relative to stimulated wild-type T cells (Carpino et al., 2009; Carpino et al., 2004; San Luis et al., 2011; Tsygankov, 2013). Interestingly, in primary CD4⁺ and CD8⁺ naïve splenic T cells lacking the Sts proteins, increased levels of phosphorylation are visible on both the native 70 kDa form of Zap-70 and on slower migrating forms that are recognized by antibodies to ubiquitin (Carpino et al., 2004). Recently, it was also demonstrated that Zap-70 is modified by K33-linked polyubiquitination predominantly on Lys-578 following TCR engagement, and that the Sts proteins play a role in the dephosphorylation of polyubiquitinated Zap-70 in stimulated CD8⁺ T cells (Yang et al., 2015). Together, these results demonstrate that

ubiquitination of Zap-70 is likely a mechanism involved in the regulation of signaling pathways important for T cell activation.

In this study, we identify additional residues of Zap-70 that are subject to stimulation-dependent ubiquitination, and demonstrate a functional role for ubiquitination of Zap-70 Lys-217 in the negative regulation of TCR signaling pathways.

3. Results

3.1 Ubiquitination of Zap-70 following TCR stimulation

To determine whether Zap-70 ubiquitination could be observed in multiple cell types, we treated human Jurkat T cells and murine TK-1 T cells for 2 minutes with TCR stimulatory antibodies, isolated Zap-70 by immunoprecipitation, and assessed levels of post-translationally modified Zap-70 via immunoblot analysis. Figure 1A illustrates that phosphorylated Zap-70 isolated from both a human and mouse T cell lines following TCR stimulation migrates by SDS-PAGE in a characteristic laddering pattern that is suggestive of ubiquitin modification. Treatment of cells with pervanadate, a phosphatase inhibitor, led to significantly increased levels of dually modified Zap-70 relative to non-pervanadate treated cells. We also observed a similar pattern of post-translational modification in primary splenic T cells that were isolated from wild-type mice, cultured *ex vivo* for 4 days, and then treated with TCR stimulatory antibodies (Fig 1B, *left*). Finally, Zap-70 isolated from stimulated human T cells isolated from peripheral blood also displayed a similar pattern of post-translational modification (Fig. 1B, *right*).

Treatment of murine T cells with the phosphatase inhibitor pervanadate over a 10 minute time course of stimulation led to significantly increased levels of phosphorylation on both the 70kDa form of Zap-70, as well as its slower migrating forms (Fig. 2A). Thus, accumulation of dually modified forms of Zap-70 occurs rapidly following TCR stimulation and inhibition of phosphatase activity increases the relative abundance of the dually modified forms. In contrast, treatment of T cells with proteasome inhibitor did not alter the levels of accumulation of dually modified Zap-70. For example, as illustrated in Figure 2B, levels of modified Zap-70 peaked between 2 and 5 minutes following TCR stimulation and then gradually declined in a manner that was unaffected by MG-132 treatment. Thus, we observed no difference in the levels of modified Zap-70 in cells treated with either pervanadate alone or cells treated with both pervanadate and MG-132 (Fig. 2C). The failure of MG-132 treatment to visibly enhance accumulation dually modified Zap-70 suggests that ubiquitinated Zap-70 is not degraded by the proteasome.

3.2 Identification of Zap-70 ubiquitination sites

Global ubiquitome analysis (see Materials and Methods) of unstimulated and stimulated *Sts-1/2^{-/-}* T cells identified ten ubiquitination sites in Zap-70 (Table 1). All identified sites displayed an increase in the level of ubiquitin modification following stimulation, with the largest increases (>20 fold) observed for Lys-193, Lys-217, and Lys-376 (Table 1). The remaining identified sites of ubiquitination displayed significantly reduced fold increase in stimulation-dependent modification. We also purified dually modified Zap-70 from

stimulated cells by combining affinity-based enrichment of tyrosine phosphorylated proteins with an immunoprecipitation step that isolated Zap-70 from the subsequent pool of phosphorylated proteins (see Materials and Methods). This approach yielded phosphorylated Zap-70 that was immunoreactive to antibodies against pZap-70 Tyr-493 and ubiquitin (Fig. S1). Mass spectrometry analysis of purified proteins confirmed the two major ubiquitination sites that were identified in the global analysis, Lys-193 and Lys-217 (Fig. S2 and S3).

Of the ten Zap-70 ubiquitination sites that were identified in stimulated primary T cells, only two are located in the kinase domain (Fig. 3). In particular, Lys-376 is located within the α C helix of the N-lobe of the kinase domain, while Lys-541 is located between the α G helix and α H helix of the kinase domain C-lobe (Fig. 3B). Interestingly, the majority of sites (60%) are located within the tandem SH2 domains, in the region of Zap-70 that interacts with the phosphorylated ITAMs of the TCR/CD3 subunits during T cell activation (Fig. 3C). Lys-25, Lys-75 and Lys-100 are located in the N-terminal SH2 domain, while Lys-193, Lys-206, and Lys-217 are within the C-terminal SH2 domain. Lys-193 is adjacent to three residues (Arg-170, Arg-190, and Arg-192) within the Zap-70 phospho-tyrosine binding pocket that have been demonstrated to make direct hydrogen bonding contact with a phosphate moiety of the activated ITAM (Hatada et al., 1995). Lys-217 is located adjacent to the Zap-70-ITAM binding surface, at the interface of the two SH2 domains. Lys-132 is located in Interdomain A, which links the two SH2 domains, and Lys-328 is within Interdomain B, which connects the C-terminal SH2 domain and the kinase domain (Au-Yeung et al., 2009). All ten sites are evolutionarily conserved (Fig. S4). Based on the large stimulation-dependent fold increase of the Lys-193, Lys-217, and Lys-376 modification sites, we chose these three sites for further functional analysis, although it should be noted that the relative abundance of modified Lys-376 was significantly lower than the relative abundance of either Lys-193 or Lys-217 (Table 1).

3.3 Increased pathway activation in the absence of the Zap-70 Lys-217 ubiquitination site

The P116 Jurkat cell line lacks expression of Zap-70, rendering cells unresponsive to TCR stimulation (Williams et al., 1998). To evaluate the role of Zap-70 ubiquitination in the regulation of signaling pathways downstream of the TCR, we generated P116 Jurkat cell lines stably expressing wild-type Zap-70 or Zap-70 Lys-to-Arg mutants in which ubiquitin-acceptor lysines were replaced by an arginine, thereby blocking ubiquitination at the specific site. Expression level of Zap-70 was comparable in each stable cell line (Fig. S5). Further, the recruitment of wild-type Zap-70 and Zap-70 SH2 domain mutants into the activated TCR complex was comparable, as evinced by the equivalent levels of Zap-70 associated with CD3- ζ following stimulation (Fig. S6). To assess levels of Zap-70 activation following TCR stimulation, cells were stimulated and levels of activation loop tyrosine phosphorylation were evaluated by western blot. We consistently noted increased levels of phosphorylated Tyr-493 in cells expressing Zap-70 K217R single mutant and K193/217R double mutant (Fig. 4A). In contrast, no difference in Zap-70 activation levels was observed when Lys-193 or Lys-376 were individually altered to prevent ubiquitin modification. These results suggest that ubiquitination of Zap-70 Lys-217 following TCR stimulation plays a negative regulatory role with regard to phosphorylation and activation of Zap-70.

A kinetic analysis of kinase activation following TCR stimulation revealed that cells expressing Zap-70 K217R display significantly enhanced levels of activated Zap-70 at early time points (within 2 minutes), but the differences between levels of activated Zap-70 in WT- and K217R-expressing cells become insignificant by 5 minutes after stimulation (Fig. 4B, *top*). This analysis also revealed that the lack of an effect on cells expressing Zap-70 K193R is not due to a shift in the time of maximal activation of Zap-70, as the levels of activated Zap-70 in cells expressing K193R are similar to wild-type at both earlier (2 min) and later (10 min) time points (Fig. 4B, *bottom*). These results suggest that ubiquitination of Zap-70 Lys-217 is an acute response that limits Zap-70 activation following TCR stimulation.

We then assessed signaling events downstream of Zap-70. The adaptor proteins LAT and SLP-76 are two important substrates of Zap-70 whose phosphorylation is critical for activation of downstream pathways (Koretzky and Myung, 2001; Malissen et al., 2014). The stimulation-dependent phosphorylation of both LAT and SLP-76 (Fig. 5A, B) was found to be enhanced in P116 cells expressing Zap-70 K217R and Zap-70 K193R/K217R relative to cells expressing wild-type Zap-70, Zap-70 K193R, or Zap-70 K376R. Downstream of activated adaptor proteins, levels of phosphorylated MAPK following TCR stimulation were also enhanced in P116 cells expressing Zap-70 K217R and Zap-70 K193R/K217R compared to cells expressing wild-type, K193R, and K376R Zap-70 (Fig. 5C). These results suggest that the enhanced tyrosine phosphorylation of Zap-70 observed in the absence of the Lys-217 ubiquitin modification site results in increased activation of downstream signaling pathways.

3.4 Increased IL-2 production in the absence of Zap-70 Lys-217 ubiquitination site

The functional outcome of early signaling events following TCR engagement can lead to T cell activation, including T cell proliferation, differentiation and effector cytokine secretion. The induction of IL-2 expression is one of the hallmarks of T cell activation following TCR ligation (Smith, 1988). Because the Zap-70 K217R mutant consistently displayed enhanced activation of proximal elements of the TCR pathway, we sought to determine whether increases in pathway activation observed in the absence of the Lys-217 ubiquitin modification site resulted in downstream functional effects. Therefore, we compared levels of IL-2 secretion in P116 cells stably expressing wild-type Zap-70 and the K217R mutant. We detected increased IL-2 secretion in cells expressing Zap-70 K217R relative to cells expressing wild-type Zap-70 following stimulation with ConA (Fig. 6A) and following co-incubation with Raji cells in the presence of SED (Fig. 6B). We also compared levels of IL-2 expression in primary T cells that over-expressed wild-type Zap-70 or the K217R mutant and noted increased IL-2 expression in the latter relative to the former (Fig. 7A, B), despite similar levels of kinase expression (Fig. S7). These results suggest that the enhanced activation of TCR signaling pathways observed in the absence of the Lys-217 ubiquitin modification site results in increased transcriptional activation, including expression of the cytokine IL-2.

4. Discussion

Protein ubiquitination is a highly versatile, dynamic, and functionally important post-translational modification. Indeed, it is well established that post-translational modification of a protein substrate by either a single ubiquitin polypeptide or long chains of polymeric ubiquitin can alter a protein's stability, activity, localization, and interacting partners (Komander and Rape, 2012). While the role of ubiquitination in targeting protein substrates for proteasome-mediated degradation has been extensively characterized, the additional functional consequences of ubiquitin modification have emerged more recently (Komander and Rape, 2012). This study supports the hypothesis that Zap-70 ubiquitination is a mechanism for regulating Zap-70 function downstream of the TCR. The ubiquitination of Zap-70 was first observed in mutant T cells lacking the Sts regulatory proteins, due to the hyper-phosphorylation of both native Zap-70 and its ubiquitin-modified forms in the absence of the Sts phosphatases (Carpino et al., 2004). An important question that arose from this observation was whether Zap-70 ubiquitination was limited to *Sts-1/2*^{-/-} T cells, in which the normal biochemical pathways that control TCR signaling are deregulated. Our current study demonstrates that ubiquitination of Zap-70 following T cell stimulation is a phenomenon that can be observed in human and mouse T cell lines and primary human and murine T cells. This suggests it could be part of the normal biochemical processes that regulate the extent of T cell activation following antigen stimulation. This hypothesis is supported by recent evidence that the Nrdp1 E3 ubiquitin ligase targets Zap-70 for K33-linked ubiquitination in CD8⁺ T cells (Yang et al., 2015).

Our analysis identified 10 lysines within Zap-70 that are sites of ubiquitination, three of which displayed greater than 20-fold increase in levels of modification following TCR stimulation. We currently do not know the stepwise series of biochemical events that induce or promote Zap-70 ubiquitination at the varied lysine residues, or whether the recruitment of Zap-70 to the TCR and its subsequent activation by phosphorylation are necessary intermediate signaling steps to the activation of E3 ligases such as Nrdp1 that target Zap-70. Six of the identified ubiquitination sites are located within the Zap-70 N-terminal SH2 domains (see Table 1). The N-terminal region mediates interaction of the kinase with phosphorylated ITAMs of the activated TCR complex, and we surmise that steric constraints could make ubiquitin post-translational modification of Zap-70 incompatible with its localization in the TCR complex. Thus, it is not clear whether Zap-70 is ubiquitinated while it is bound to TCR subunits. We favor a stepwise model in which Zap-70 recruitment to the activated receptor complex, its subsequent phosphorylation by Lck, and its activation of downstream signaling cascades precede the activation of a negative feedback loop that results in Zap-70 ubiquitination and functional inhibition. It is also important to note that the E3 ubiquitin ligase(s) involved in modification of the identified residues is (are) not known. While the well-studied Cbl family of E3 ligases have been demonstrated to interact with Zap-70, the latter is thought to act primarily as an adaptor protein that recruits Cbl to the activated receptor complex (Rao et al., 2002; Wang et al., 2001). Indeed, the targets of the Cbl ligases are considered to be the TCR, Lck, and the p85 subunit of PI3K, and no study to date has linked their activity with direct Zap-70 ubiquitination (Dufour et al., 2008; Rao et al., 2002; Wang et al., 2001). Nrdp1 was recently identified as an E3 ligase that targets six

sites within Zap-70, with Lys-578 as the main ubiquitin acceptor site (Yang et al., 2015). Of the sites identified in the latter study, only one (Lys-328) matched with the lysine acceptor sites identified in our study. This discrepancy might be due to the differences in cell types or methods used in the two studies. For example, Yang et. al. identified modified lysine residues following co-expression of Zap-70 and Nrdp1 in HEK 293T cells. In contrast, we determined TCR stimulation-induced ubiquitination sites on Zap-70 isolated from stimulated primary *Sts-1/2^{-/-}* T cells. Given the large number of Zap-70 ubiquitination sites and their distinct locations on the surface of the molecule, an interesting possibility is that multiple E3 ligases target distinct sites within Zap-70 for ubiquitination in a spatially and temporally distinct manner.

Zap-70 function in T cell activation is regulated in part by multiple phosphorylation events, and we observed Zap-70 that lacks the Lys-217 ubiquitination site displayed a marked increase in levels of activation following TCR stimulation relative to wild-type Zap-70. Increased phosphorylation of downstream molecules LAT, SLP-76, and MAPK was also evident in cells reconstituted with the K217R mutant. Increased activation of signaling pathways in K217R – expressing Jurkat P116 cells was accompanied by increased IL-2 production following TCR stimulation. Interestingly, we also observed that over-expression of Zap-70 K217R in primary T cells led to increased levels of IL-2 expression downstream of the TCR, as represented by greater numbers of responding cells. This is consistent with observations that T cells respond to stimulation by undergoing digital all-or-nothing response and increasing stimuli leads to increased percentage of responding cells (Wertek and Xu, 2014). Together, these results suggest that Zap-70 ubiquitination at Lys-217 acts in a non-redundant fashion to negatively regulate Zap-70 kinase activity. In particular, these results are consistent with a role for ubiquitination in regulating the phosphorylation and level of activation of Zap-70. One possibility is that ubiquitination at Lys-217 creates a binding site for regulatory molecules that directly or indirectly target Zap-70 phosphorylation sites. An intriguing question yet to be resolved is whether simultaneous modification of Zap-70 by tyrosine phosphorylation and ubiquitination has specific functional consequences. Ubiquitination of residues adjacent to phosphorylation sites could alter conformational changes induced by phosphorylation, or restrict the formation of multi-protein signaling complexes by interfering with phosphorylation-induced recruitment of signaling molecules. Addressing these questions will add a new dimension to our understanding of the role of Zap-70 in regulating T cell activation.

Materials and Methods

Mice

The generation of mice containing the *Sts* mutations, backcrossed 10 generations onto the C57/B6 background, has been described (Carpino 2004). Mice were housed and bred in the Stony Brook University Animal Facility under specific pathogen-free conditions. Mice used for experiments were 8 weeks old. All mice were maintained in accordance with Stony Brook University Division of Laboratory Animal Resources (DLAR) guidelines. All animal experiments were approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC).

Cell culture

Raji B cells (ATCC CCL-86), Jurkat clone E6-1 (ATCC TIV-152), P116 (Zap-70 deficient Jurkat sub-clone; ATCC CRL-2676), and P116 cells expressing wild type or mutant Zap-70 were maintained at 37°C in 5% CO₂ in RPMI (Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 µM β-mercaptoethanol, and the appropriate concentration of penicillin and streptomycin. TK-1 cells (ATCC CRL-2396) were cultured in T cell medium (RPMI media containing 10% FBS, 10 mM HEPES (pH 7.0), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 0.1 mM nonessential amino acids, and the appropriate concentration of penicillin/streptomycin). HEK 293T cells (ATCC CRL-11268) were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. Primary T cells were derived from wild-type and *Sts-1/2^{-/-}* mice by crushing spleens in PBS containing 2% FBS, lysing red blood cells in ACK lysis buffer (150 mM NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA), and removing debris by filtration through a 70 µm filter (BD). T cells were expanded for 2 days in T cell medium supplemented with 0.5 µg/ml anti-CD3ε antibody (145-2C11) and 10 U/ml IL-2. T cells were further expanded for 2 days in fresh T cell media containing IL-2.

Antibodies and reagents

Antibodies against Zap-70 (A-1), SLP-76 (6A462), LAT (FL-233), CD3-ζ (6B10.2), and ubiquitin (P4D1) were obtained from Santa Cruz Biotechnology. Anti-pTyr (4G10) antibody was from Upstate Biotechnology, Inc. Polyclonal antibodies against phospho-Zap-70 (Tyr-493), SLP-76, MAPK, and phospho-MAPK (Thr 202/Tyr 204), and monoclonal MAPK (3A7) antibody were obtained from Cell Signaling Technology. Anti-β-actin (8H10) antibody was from Applied Biological Materials, Inc. Anti-Flag (M2) antibody was purchased from Sigma-Aldrich. For immunoblotting analysis, secondary antibodies were Alexa Fluor® 680-conjugated goat anti-mouse (Molecular Probes) and IRDye800-conjugated goat anti-rabbit (LI-COR). Agarose-conjugated phospho-tyrosine antibody (4G10) used in purification of ubiquitinated Zap-70 was obtained from EMD Millipore. Fluorochrome-conjugated IL-2 antibody (JES6-5H4) used for intracellular cytokine staining was purchased from BioLegend. Fluorochrome-conjugated Thy1.2 antibody (53-2.1) was purchased from eBioscience. For stimulation of murine T cells, anti-CD3ε (purified 145-2C11 or biotinylated 500A2), biotinylated anti-CD4 (RM4-5), and biotinylated anti-CD8 (53-6.7) antibodies were employed. For stimulation of human T cells, biotin-conjugated CD3ε (UCHT1) antibody (Ansell) was used. FITC-streptavidin (BD Pharmingen) was used for cross-linking. Recombinant human IL-2 cytokine was purchased from PeproTech Inc. (Rocky Hill, NJ). Partially purified Staphylococcal enterotoxin D (SED) was obtained from Toxin Technology (Sarasota, FL).

Cloning and site-directed mutagenesis

All cDNA mutants were constructed by PCR mutagenesis using full-length murine Zap-70 coding sequence (Genbank Acc. No. U77667.1) and sequenced to confirm the presence of only the desired mutation.

Generation of stable cell lines

HEK 293T cells were co-transfected with a pCDH-EF1-MCS-T2A-copGFP lentiviral expression vector containing mutant Zap-70, along with packaging (pPACKH1-Gag and pPACKH1-Rev) and envelope (pVSV-G) plasmids (System Biosciences). Supernatant was collected from transfected cells 48 and 72 hrs post-transfection and used to spin-infect P116 cells in the presence of 8 µg/ml Polybrene (EMD Millipore). GFP⁺ cells were isolated using a FACSaria flow cytometer (BD Biosciences) and Zap-70 expression was verified by Western blot.

T cell stimulation

Primary murine T cells (6×10^7) were labeled on ice for 25 min with 20 µg/ml biotin-conjugated anti-CD3ε (500A2), anti-CD4 (RM4-5) and anti-CD8a antibodies (53-6.7), washed with PBS with 2% FBS, incubated on ice for 25 minutes with 10 µg/ml FITC-streptavidin in the presence or absence of pervanadate as indicated, placed in a 37°C water bath for 2 minutes or for the indicated times, and immediately lysed in cold lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing 100 µM PMSF, Complete™ protease inhibitors (Roche), 1 mM Na₃VO₄ and 25 mM N-ethylmaleimide (Sigma). A stock solution of 50 mM pervanadate was freshly prepared by mixing equal volumes of 100 mM H₂O₂ with 100 mM Na₂VO₄. For proteasomal inhibition, cells were pre-treated with 100 µM MG-132 (Boston Biochem, Inc.) for 1 hr at 37°C, then stimulated as described with MG-132 present during all the steps of the stimulation. Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll-Hypaque (Lymphocyte Separation Medium 1.077, Lonza) gradient centrifugation. Peripheral blood T cells were isolated from PBMC by negative selection using Pan T cell Isolation Kit (Miltenyi Biotec). Primary human T cells (3.5×10^7) were stimulated as described above using biotin-conjugated CD3ε (UCHT1) antibody and FITC-streptavidin.

Immunoprecipitation and immunoblotting

Cell extracts were prepared in ice-cold lysis buffer, clarified by centrifugation and rotated at 4°C with specific antibody and Protein A-sepharose beads (Sigma) for 2 hrs. After beads were washed 3 times in lysis buffer, bound proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane (Whatman). Membranes were probed with specific antibody followed by the appropriate secondary antibody and developed with the Odyssey Infrared Imaging System (LI-COR) or Odyssey CLx Imaging System (LI-COR). Densitometry analysis of Western blots was performed using Image Studio Lite 4.0 software (LI-COR).

Identification of Zap-70 ubiquitination sites

Activated *Sts-1/2^{-/-}* T cells were stimulated with anti-CD3 antibody for 2 minutes at 37°C and lysed as described above. Cell lysates were clarified by centrifugation and flowed over a column of agarose-conjugated phospho-tyrosine antibody (4G10). Bound proteins were eluted from the column with 3% acetic acid containing 0.5% SDS. Eluted fractions were applied to Amicon® Ultra-4 centrifugal filter units (3 kDa MWCO, EMD Millipore) for

filtration and buffer exchange into a modified lysis buffer containing 0.01% RapiGest™ SF Surfactant (Waters), followed by Zap-70 immunoprecipitation with pZap-70 Y493 antibody and protein A magnetic beads (EMD Millipore). Beads were flash-frozen in dry ice-ethanol bath prior to UbiScan® analysis (Cell Signaling Technology, Inc.). For quantitative profiles of ubiquitinated proteins comparing unstimulated and stimulated cells, splenocytes were isolated from spleens of *Sts-1/2^{-/-}* mice and activated in culture. Unstimulated cells and cells stimulated with anti-CD3 antibody for 2 minutes at 37°C were flash-frozen in a dry-ice ethanol bath prior to UbiScan® analysis. For the latter, peptide preparation, immunoprecipitation, and MS analysis were performed as described (Stokes et al., 2012). MS/MS spectra were evaluated using SEQUEST 3G and the SORCERER 2 platform from Sage-N Research (v4.0, Milpitas CA) (Lundgren et al., 2009).

Retroviral infection and intracellular cytokine analysis

After 24 hr growth in the presence of 0.5 µg/ml anti-CD3 antibody (145-2C11) and 10 U/ml IL-2, wild-type splenocytes were spin-infected with a retrovirus carrying a bicistronic cassette expressing wild-type or mutant Zap-70 upstream of an IRES-GFP element (Persons et al., 1997). Infected T cells were cultured for 48 h in the presence of IL-2, stimulated with 1 µg/ml plate-bound anti-CD3 antibody (145-2C11) for 6 hrs in the presence of 5 µg/ml Brefeldin A (Cell Signaling Technology, Inc.) and stained for Thy1.2 (53-2.1) and intracellular IL-2 (clone JES6-5H4) using Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). GFP⁺ cells were analyzed for IL-2 expression using a BD FACSCalibur flow cytometer and FlowJo software (Tree Star, Inc.).

IL-2 quantification

P116 cells stably expressing wild type or mutant Zap-70 were grown in serum-free media for 4 hrs and then stimulated in culture for with 25 µg/ml Concanavalin A (Sigma-Aldrich) or mixed with equal number Raji cells alone or Raji cells and 100 ng/ml Staphylococcal enterotoxin D (SED; Toxin Technology) (Shapiro et al., 1998). Cell culture supernatants were collected after 24 hours and IL-2 assayed by ELISA with Human IL-2 ELISA Max Deluxe kit (BioLegend) using VersaMax Microplate Reader with SoftMax Pro Software (Molecular Devices).

Statistical analysis

Quantitative data for all experiments are expressed as mean ± standard deviation (SD) for each group. Data were analyzed using Prism Software (GraphPad). Unpaired two-tailed Student's t-test was used for all data analysis and P-values <0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TCR	T cell receptor
Zap-70	Zeta-chain associated protein of 70 kDa

Highlights

- Zap-70 is ubiquitinated following TCR stimulation.
- We identified ten ubiquitination sites in Zap-70 by mass spectrometry.
- Disruption of ubiquitin acceptor Lys-217 results in increased Zap-70 signaling activity.

Figure 1. Ubiquitination of Zap-70 following TCR stimulation

A. Jurkat T cells (*left*) or TK-1 T cells (*right*) were treated with pervanadate as indicated and stimulated with CD3 antibodies. The stimulated cells were lysed and processed for IP/ Western analysis to visualize levels of Zap-70. Immunoprecipitated Zap-70 was assessed for levels of phospho-Zap-70 Y493 (*top*), total Zap-70 (*middle*), and ubiquitin (*bottom*). **B.** Splenocytes from wild type mice (*left*) and primary human T cells (*right*) were treated with pervanadate as indicated and stimulated with CD3 antibodies. Following Zap-70 immunoprecipitation, levels of phospho-Zap-70 Y493 (*top*), total Zap-70 (*middle*), and ubiquitin (*bottom*) were assessed.

Figure 2. Levels of ubiquitinated Zap-70 in stimulated T cells are unaffected by proteasome inhibition

Splenocytes from wild type mice were left untreated, treated with pervanadate (**A**), MG-132, or treated with both MG-132 and pervanadate (**B**), following which they were stimulated, lysed and processed for IP/Western analysis. Immunoprecipitated Zap-70 was assessed for levels of phospho-Zap-70 Y493 (*top*) and Zap-70 (*middle*). Whole-cell lysates were separated by SDS-PAGE and subjected to Western blot analysis with anti-ubiquitin antibody (*bottom*). Representative blots are illustrated (**A,B**). **C.** Histogram plot shows densitometry analysis of Western blot and represents optical density of the entire lanes between 75 kDa and 170 kDa. Levels of dually modified Zap-70 were quantified and normalized to total Zap-70 in each lane (including 70 kDa form). All sets are shown as percent of levels in untreated cells stimulated for 2 min (*lane 2*). Data represented as mean $\pm$ SD for at least two (**A**) or three (**B**) independent experiments. No statistically significant difference was found between untreated and MG-132 treated samples, or between cells treated with MG-132 and pervanadate and those treated with pervanadate alone.

Figure 3. Zap-70 ubiquitination sites

A. Schematic representation of full-length Zap-70. Arrows indicate location of ubiquitination sites. Ubiquitination sites identified by Yang et. al. 2015 are marked with (▼) (Yang 2015). Phosphorylation sites are indicated with (*). **B.** Crystal structure of full length inactive Zap-70 (PDB ID: 2OZO). The N-terminal and C-terminal SH2 domains, interdomain A, interdomain B, and kinase domain are shown in yellow, orange, green, red, and blue, respectively. Ubiquitination sites identified by UbiScan analysis are indicated in dark gray. **C.** Crystal structure of Zap-70 tandem SH2 domains in complex with a peptide derived from the first ITAM of the CD3- ζ chain (PDB ID: 2OQ1). CD3- ζ peptide is shown in cyan. Ubiquitination sites identified by UbiScan analysis are shown in dark gray.

Figure 4. Increased levels of activated Zap-70 in the absence of Zap-70 Lys-217 ubiquitination site

A. P116 cells stably expressing the indicated Zap-70 Lys-to-Arg mutants were stimulated with CD3 antibody for 2 minutes, lysed and processed for IP/Western analysis. **B.** P116 cells stably expressing WT Zap-70 and K217R (*top*) or K193R (*bottom*) mutants were stimulated with CD3 antibody for the indicated time, lysed and processed for IP/Western analysis. Levels of phosphorylated Zap-70 were assessed. Representative results for are displayed (*left panel*). Histograms (*right panel*) show densitometry analysis of Western blots. Relative phosphorylation of Zap-70 in stimulated cells was normalized to total Zap-70 for each sample and shown as percent of wild-type levels. Data represented as mean $\pm$ SD for at least three independent experiments (* p <0.05, ** p <0.01, *** p <0.001 vs. WT; Student's t-test).

Figure 5. Enhanced signaling in the absence of Zap-70 Lys-217 ubiquitination site

P116 cells stably expressing the indicated Zap-70 Lys-to-Arg mutants were stimulated with CD3 antibody for 2 minutes, lysed and processed for IP/Western analysis. Levels of phosphorylated LAT (A), SLP-76 (B), and activated MAPK (C) were assessed as indicated. Representative results for each signaling molecule are displayed (*left panel*). Histograms (*right panel*) show densitometry analysis of Western blots. Relative phosphorylation of the indicated proteins in stimulated cells was normalized to total protein for each sample and shown as percent of wild-type levels. Data represented as mean $\pm$ SD for at least three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. WT; Student's t-test).

Figure 6. Enhanced IL-2 production in P116 cells stably expressing Zap-70 lacking the Lys-217 ubiquitination site

P116 cells stably expressing wild-type Zap-70 or Zap-70 K217R mutant were stimulated with Concanavalin A (A) or Raji/SED (B). After 24 hours in culture, supernatants were collected and assayed for IL-2 content by ELISA. Results are representative of three independent experiments for each condition (mean $\pm$ SD; ** $p < 0.01$ vs. WT; Student's t-test).

Figure 7. Enhanced IL-2 expression in primary cells over-expressing Zap-70 lacking the Lys-217 ubiquitination site

Primary T cells were infected with empty vector, or retrovirus expressing wild-type Zap-70 (WT) or Zap-70 K217R mutant. Infected cells were stimulated with CD3 antibody (1 μ g/ml) for 6 hours. Following stimulation, cells were stained with fluorochrome-labeled antibodies to Thy1.2 (T cell marker) and IL-2 and analyzed by flow cytometry to assess levels of IL-2 expression. Cytokine-expressing T cells are visible in the upper right-hand quadrant with the percentage of cells indicated. Levels of IL-2-expressing T cells are indicated. Representative plots (**A**) and the summary data of three independent experiments (**B**) are illustrated (mean $\pm$ SD; * p <0.05 vs. WT).

Table 1**Ubiquitination sites in Zap-70**

Lysine residue position within Zap-70 identified as a site of ubiquitin modification, fold change in ubiquitination between stimulated and unstimulated samples, and sequence of the ubiquitinated peptide (modified lysines are marked with *). Average peak intensity is the parent ion intensity observed at its chromatographic apex and is used to determine the fold increase in ubiquitination following stimulation at each site.

		Average Peak Intensity		
Site	Fold change	Unstimulated	Stimulated	Peptide
25	6.6	5,415,628	27,978,322	AEAEHLK*LAGMADGLFLLR
75	4	5,431,784	16,922,299	QLNGTYAIAGGK*AHC GPAELCQFYSDPDGLPCNLR
100	5.9	3,367,094	15,508,399	K*PCNRPPGLEPQPGVFDCLR
132	8.9	12,820,362	89,789,764	QTKW*LEGDALEQAISQAPQVEK
193	20.8	17,238,167	279,258,112	K*EQGTALSLVYGK
206	2.6	13,720,480	27,763,319	KEQGTALSLVYGK*TVYHYLISQDK
217	19.4	19,639,150	295,996,576	TVYHYLISQDK*AGK
328	4.1	1,086,215	3,477,448	LASSTDKPRPMPDTSVYESPYSDPEELKDK*K
376	23.6	575,780	10,600,356	QGTEK*ADKDEMMR
541	2.9	1,783,042	4,050,919	K*MKGPEVLDFIK