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Fine-tuning of proximal TCR signaling by ZAP-70 tyrosine residues in Jurkat cells

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Abstract

Zeta-chain-associated protein kinase of 70kDa (ZAP-70) kinase is a key regulator in the early steps of TCR signaling but some aspects of its fine regulation are still unclear. From its 31 tyrosine (Y) residues, 11 phosphorylation sites have been identified, some with activator (Y315 and Y493) or inhibitory (Y292 and Y492) and others with unknown function (Y069, Y126 and Y178). In our present work, we aimed to elucidate the role of different Y residues of ZAP-70, especially those with unknown function, in calcium signaling and the autoregulation of the kinase. ZAP-70-deficient Jurkat cells (P116) were stably reconstituted with point-mutated ZAP-70 constructs where tyrosine residues 069, 126, 178, 238, 292, 315, 492 or 493 were replaced with phenylalanine (F). The anti-CD3-elicited calcium signal increased in F069-, F292- and F492-ZAP-70-expressing cell lines but decreased in the F126-, F315- and F493-ZAP-70-expressing cell lines. ZAP-70 point mutations led to phosphorylation changes predominantly in SH2 domain containing leukocyte protein of 76kDa (SLP-76) but not linker of activated T cells (LAT) during CD3-activation; moreover, we detected basal hyperphosphorylation of SLP-76 Y128 in the F126-, F178- and F492-ZAP-70-expressing cell lines. In summary, Y069, Y178, Y292 and Y492 have inhibitory, while Y126, Y315 and Y493 activator role in anti-CD3-induced T-cell activation. Phosphorylation changes in LAT and SLP-76 suggest that fine regulation of ZAP-70 on calcium signaling is rather transmitted through SLP-76 not LAT. Additionally, negative or positive autoregulatory function of Y292 and Y493 or Y315, respectively, was revealed in ZAP-70. These data indicate that previously not characterized Y069, Y126 and Y178 in ZAP-70 participate in the fine regulation of TCR signaling.

Keywords: lentiviral transfection, site-directed mutagenesis, TCR signaling, ZAP-70

Introduction

T cells are key players of adaptive immunity: they recognize peptide antigens with their TCR in an MHC-restricted manner (1, 2), which leads to their activation/differentiation and the engagement of effector mechanisms. Co-receptors, like CD4 or CD8, CD28 and the protein tyrosine phosphatase CD45 are also involved in TCR-mediated signaling (3–5). A complex network of signaling events is prerequisite for T-cell activation. Upon close TCR–peptide–MHC binding, early phosphorylation steps are initiated. First, the sarcoma (Src) non-receptor tyrosine kinase family member, lymphocyte-specific protein tyrosine kinase (Lck) (CD4/8 associated) is primed by the phosphatase CD45 through the removal of an inhibitory phosphate group from tyrosine (Y)505 (6). Next, Lck is activated by the phosphorylation of Y394 by the activated TCR complex (6). The activated Lck, in turn, phosphorylates immunoreceptor tyrosine-based activation motifs found in the TCR-associated CD3 complex (7). The phosphorylated CD3 ζ chain provides a docking site for the spleen tyrosine kinase (Syk) family member zeta-chain-associated protein kinase of 70kDa (ZAP-70) kinase (8).

ZAP-70 is phosphorylated by Lck and activated to become a key organizer of downstream TCR signaling steps. Two important target molecules of the ZAP-70 are the adapter proteins linker of activated T cells (LAT) and SH2 domain containing leukocyte protein of 76kDa (SLP-76) (9–11). Phosphorylation of these molecules leads to the formation of a multimolecular complex involving growth factor receptor-bound protein 2 (GRB2), IL2-inducible T-cell kinase, GRB2-related adaptor downstream of Src homology 2 domain containing transforming protein (Shc) and Vav that results in activation of phospholipase C-γ1 (PLCγ1) (12, 13). PLCγ1, in turn, cleaves phosphatidylinositol 4,5-bisphosphate producing two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (14). DAG initiates two major pathways

the rat sarcoma (Ras) and proteine kinase C theta (PKCθ) signaling (15). Ras triggers the MAP kinase cascade that results in the activation of transcription factors [e.g. activator protein 1 (AP-1)] (16), while activation of PKCθ activates the nuclear factor κB (NFκB) pathway leading also to transcriptional regulation (17).

IP_3 releases Ca^{2+} from the endoplasmic reticulum (intracellular Ca^{2+} store) that is followed by the opening of plasma membrane Ca^{2+} channels as well (capacitative influx) (18). Elevated intracellular Ca^{2+} level then activates calcineurin, calmodulin and finally the transcription factor nuclear factor of activated T cells (NFAT) (19). As a consequence of all above-mentioned signaling cascades, a number of transcription factors are activated (AP-1, NFAT and NFκB) which lead to complex gene expression changes in activated T cells (20).

The ZAP-70 kinase is member of the Syk non-receptor tyrosine kinase family and is expressed in T cells, NK cells and basophile granulocytes (8, 21). ZAP-70 consists of two N-terminal Src homology 2 (SH2) domains and a C-terminal kinase domain separated by interdomains A and B, respectively (22). ZAP-70 contains 31 Y residues in total, 11 of which have been identified as phosphorylation sites by mass spectroscopy; however, the physiological function of some is still unknown (23). For example, little is known about Y069, Y126, Y178 and Y238, located in the two SH2 domains and interdomain A, although Y126 is considered to be involved in the autophosphorylation of the kinase (23). Interdomain B contains three regulatory Y residues at 292, 315 and 319. Y292 plays an inhibitory role in TCR-mediated signaling and serves as docking site for casitas B-lineage lymphoma (24–26). Y315 binds Vav and sarcoma virus CT10 oncogene homolog II and bears both positive and negative regulatory functions in T-cell activation (27, 28). Y319 plays a positive regulatory function and is important in PLC-γ1 and Ras-mediated signaling (29, 30). Y residues found in interdomain B also influence T-cell development (31–33). Five tyrosines of the kinase domain are considered to take part in T-cell activation. Y474 is the docking site for Shc (34); Y492 has inhibitory, while Y493 has an activatory role in T-cell activation (35, 36). Y597 and Y598 are negative regulatory sites (37). Most of the functions of specific Y residues mentioned so far were examined in either B cell-based systems or Jurkat cells.

In our present work, we aimed to elucidate the function yet unknown Y residues of the ZAP-70 kinase. To that end, we reconstituted P116 cells (a ZAP-70-deficient Jurkat subclone) with point-mutated ZAP-70 molecules, where Y residues at positions 069, 126, 178, 238, 292, 315, 492 or 493 were replaced with phenylalanine (F) using a lentiviral transfection system. After establishing the mutant ZAP-70-expressing cell lines, we analyzed how these mutations affected the TCR signaling pathway by characterizing the Ca^{2+} signaling and phosphorylation events. Two important substrates of the ZAP-70 kinase, LAT and SLP-76 molecules, were studied in more detail. Here, we provide new data about the regulatory functions of Y069, Y126, Y178 residues of the ZAP-70 kinase. Moreover, we show that ZAP-70 point mutations exert their regulatory effect on Ca^{2+} signaling through SLP-76. Importantly, we established an array of new Jurkat cell lines carrying targeted mutations, which provide a useful tool for future signaling studies as well.

Methods

Chemicals and buffers

All fine chemicals were obtained from Sigma unless otherwise stated.

Cell lines

Jurkat (ATCC TIB-152), P116 (ZAP-70-deficient Jurkat subclone; ATCC CRL-2676) or transgenic P116 cells transfected with the wild-type (WT) or point-mutated ZAP-70 were cultured under conventional conditions (37°C, humidified atmosphere, containing 5% CO_2) in RPMI supplemented with 10% FCS (Gibco), sodium pyruvate (1 mM) and glucose (4.5 g l^{-1}), penicillin and streptomycin.

Cloning and site-directed mutagenesis of the human ZAP-70

The full-length human ZAP-70 coding sequence (henceforth WT-ZAP-70) was amplified using the primers P1 (forward) and P2 (reverse) (annealing temperature: 57°C; product length: 1881 bp) containing BamH1 and Sal1 restriciton sites, respectively (Supplementary Table 1 is available at *International Immunology* Online). We used a cDNA library transcribed from human peripheral T cell total RNA as template for the cloning PCR. Next, the PCR product was purified and cloned into a TA vector using the InsTAclone PCR cloning kit (Fermentas) according to the manufacturer's instructions; thereafter, the plasmid with the insert was sequenced.

Site-directed mutagenesis of the human ZAP-70 was done in two steps using the TA-cloned WT sequence as a template. First, PCRs were done using primers containing the Y-F mutations at different amino acid (AA) positions (Supplementary Table 1 and Figure 1 are available at *International Immunology* Online); the following forward and reverse primer combinations were used: P1-P069, P1-P126, P1-P178, P1-P238, P1-292, P315-P2, P492-P2 and P493-P2 (Supplementary Table 1 and Figure 1 are available at *International Immunology* Online). The PCR products were purified and used as megaprimers in the second step PCRs in pair with P1 or P2 to amplify the full-length ZAP-70 constructs containing the Y-F mutations. The final products were TA cloned and sequenced which verified the targeted mutations.

All PCRs were done with a high fidelity Proof Start DNA Polymerase (Qiagen) according to the manufacturer's instructions.

Stable transfection of the ZAP-70 constructs into ZAP-70-deficient P116 cell line using lentiviral vectors

P116 cells were transfected with the lentiviral vectors containing the mutated or the full-length WT-ZAP-70 cDNA. Briefly, point-mutated or WT-ZAP-70 cDNA has been inserted into the pWPTS lentiviral transfer plasmid under control of elongation factor-1 (EF1) promoter. This late second-generation lentiviral construct contains central polypurine tract and WPREs that increase transgene integration and expression (38, 39). For lentivirus production, an envelope construct (pMD.G),

a packaging plasmid (R8.91), and the transfer plasmid (pWPTS with EF1-ZAP-70) were transiently co-transfected by calcium–phosphate method into 293T cells pre-treated with chloroquine (1 mM final concentration). Following an overnight incubation and medium change, the supernatant of the virus producer cells was harvested after 24 h, centrifuged (2000 r.p.m., 10 min, 4°C) and filtered (0.45-μm pore size polyvinylidene fluoride-coated filters) to eliminate rough cellular debris (40). P116 cells were transfected by spinoculation (41) at MOI = 10 (MOI: multiplicity of infection or virus/cell ratio).

Antibodies

The following antibodies were used for western bloting: mouse monoclonal anti-phosphotyrosine (clone PY20, 1:5000) and anti-ZAP-70 (clone 29/ZAP-70 Kinase, 1:5000) antibodies were from BD Pharmingen (San Jose, CA, USA); mouse monoclonal anti-β-actin (clone AC-74, 1:50000) was from Sigma and rabbit polyclonal anti-SLP-76 (1:1000) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As secondary antibodies, HRP-conjugated goat anti-mouse IgG (1:1000; Hunnavix, Hungary) or anti-rabbit IgG HRP (1:1000; Pierce) were used.

For immunoprecipitation (IP), we used mouse monoclonal anti-SLP-76 (clone F-7; 2 μg per sample) antibody from Santa Cruz Biotechnology and rabbit polyclonal ZAP-70 (a kind gift from E. Monostori, University of Szeged, Hungary).

For flow cytometry, we used mouse monoclonal FITC-conjugated anti-ZAP-70 antibody (clone 2F3.2; Upstate Biotechnology) recognizing AAs 1–254 of the ZAP-70 kinase and mouse monoclonal PE-conjugated ZAP-70 antibody (clone 1E7.2; eBioscience) recognizing AAs 282–307 (Fig. 1 A). Phospho-specific PE-conjugated mouse anti-SLP-76 pY128 (clone J141-668.36.58) antibody was purchased from BD Biosciences.

Intracellular staining and flow cytometry for the detection of intracellular ZAP-70 expression

Cells (10^6) per sample were fixed in PBS containing 4% PFA for 20 min and then permeabilized in saponine buffer (PBS containing 0.1% NaN_3, 0.1% BSA and 0.1% saponine). Cells were labeled with two different antibodies recognizing two different epitopes of ZAP-70 (see above and Fig. 1A) in permeabilization buffer for 45 min on ice. Next, samples were washed twice with saponine buffer and once with PBS containing 0.1% BSA and 0.1% NaN_3.

Flow cytometric acquisition and analysis were done with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the CellQuest software. Cells were gated based on their forward scatter/side scatter parameters, 10 000 events were acquired in each sample. Mean fluorescence intensity values (MFI) were calculated based on histograms. As negative staining controls, unlabeled autofluorescent samples and isotype-matched control antibodies for each color were used.

Activation of cells

Anti-human-CD3 monoclonal antibody (OKT-3; ATCC CRL-8001; 5 μg per sample) was used for the activation of 10^6

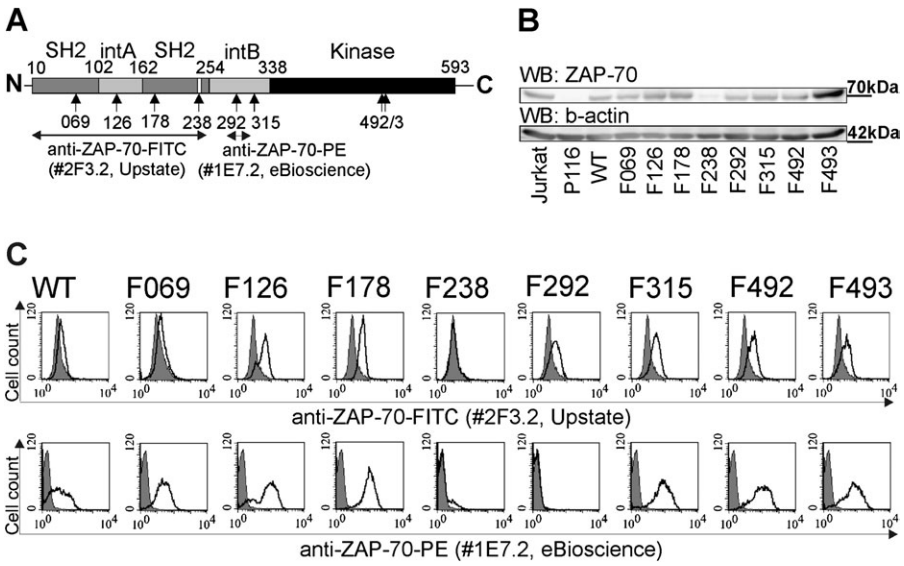

Fig. 1. Targeted point mutation analysis of selected ZAP-70 tyrosines (Y). (A) Structure of human ZAP-70. Domains of the molecule are indicated in the ribbon diagram (SH2: dark gray; interdomain: light gray and kinase: black). Numbers show the positions of selected amino acid residues. Specific Y residues that were mutated to F in the study are indicated with vertical arrows and their AA position numbers. A putative immunoreceptor tyrosine-based inhibitory motif sequence (236VEYLKL241) containing Y238 is indicated as a white area in the second SH2 domain. Epitopes of the two different monoclonal anti-ZAP-70 antibodies are indicated with horizontal double arrows. (B): ZAP-70 expression of the different cell lines was first detected with WB (upper panel). To check equal loading of the samples, we reprobed the blot with anti-β-actin antibody (lower panel). (C): ZAP-70 expression of the transfected cell lines was also analyzed with flow cytometry using two different monoclonal anti-ZAP-70 antibodies. Representative fluorescent histogram plots show the FL1 [anti-ZAP-70-FITC (#2F3.2, Upstate)] or FL2 [anti-ZAP-70-PE (#1E7.2, eBioscience)] fluorescence intensities. Gray filled histograms show the fluorescent staining of the ZAP-70-deficient P116 cells (served as negative control), whereas bold black histograms represent the fluorescent signals of the ZAP-70-transfected cells.

T cells in 100 μl RPMI, at 37°C under continuous shaking (Thermo Mixer, Eppendorf, Germany) for 2 min. For western blots (WBs), the reaction was stopped in liquid nitrogen, for Phospho-flow experiments with 4% PFA.

WB and IP

Resting or activated cells were lysed in Triton X lysis buffer (50 mM HEPES, 10 mM Na-pyrophosphate, 10 mM EDTA, 100 mM NaF, 10% glycerol and 1% Triton X-100, pH 7.3, freshly completed with protease inhibitor and Na-orthovanadate) for 30 min on ice and then centrifuged for 10 min at 13 000 r.p.m. The supernatant was either boiled in SDS sample buffer (125 mM Tris, 4% SDS, 10% glycerol, 0.006% bromophenol blue and 10% mercaptoethanol) for 10 min or further used for IP.

For IP, cell lysates were incubated in blocking buffer (Tris-buffered saline containing 10% BSA and 0.1% NaN_3) with the appropriate amount of antibody (see in Antibodies) for 2 h and then incubated with Protein-G (GE Healthcare, UK) for 2 h. After washing three times, immunocomplexes were removed from the Protein-G by 10 min boiling in SDS sample buffer.

Samples were separated on 7.5 or 10% SDS–polyacrylamide gel using a MiniProtean system (Bio-Rad, Hercules, CA, USA) and blotted onto nitrocellulose membranes with Trans-Blot equipment (Bio-Rad) overnight. Membranes were soaked in blocking buffer [2% BSA or 5% non-fatty dry milk (Bio-Rad), 10 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH 7.4] for 1 h at room temperature (RT) and then incubated with the appropriate dilution of primary antibodies (see in Antibodies) in 10-times diluted blocking buffer for an additional 2 h at RT. Washing of the membranes was performed in wash buffer (10 mM Tris, 100 mM NaCl and 0.1% Tween-20, pH 7.4). After washing, blots were developed with HRP-conjugated secondary antibodies. Following additional washing, blots were visualized using Super Signal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Signals were detected with the Fuji LAS4000 imaging system (Fuji, Japan). Antibodies were removed using Restore Western Blot Stripping Buffer (Pierce) and after blocking (see above), blots were re-probed with the second primary antibody. Densitometry of the blots was performed with the Scion-Image software (Scion Corporation, Frederick, MD, USA).

Flow cytometric detection of anti-CD3 treatment-elicited intracellular Ca^{2+} signal

To detect the changes in the free intracellular Ca^{2+} level, cells were loaded with the Ca^{2+} selective indicator dye Fluo-3-acetoxymethyl ester (Invitrogen, Carlsbad, CA, USA) according to the protocol described by Minta *et al.* (42). Flow cytometric measurements were performed and analyzed with a FACS Calibur flow cytometer using the CellQuest software. Baseline Ca^{2+} level was measured for 50 s and then OKT-3 was added to the samples and the measurements lasted for a total of 5 min. To analyze the dynamics of the Ca^{2+}signal, the changes of FL1 intensity, being proportional to the intracellular Ca^{2+} level (42), were plotted against time (43). Briefly, gates were created along the time axis of the activation dot plots at definite time points and the mean FL1 fluorescence intensities were calculated from every gate. These values were divided with the basal fluorescence intensities measured in the same sample before the addition of the activating agent and represented as 'FL-1 change' (*y*-axis) (43).

Phosphorylation changes after T-cell activation detected with Phospho-flow technique

Resting or OKT-3-activated cells were fixed with 4% PFA at 37°C for 10 min under continuous shaking and then permeabilized in Phosflow Perm Buffer III (BD Pharmingen) for 30 min on ice. Samples were then washed in PBS containing 0.1% BSA and 0.1% NaN_3 and incubated with anti-SLP-76 pY128 antibody for 45 min at RT. Samples were washed in PBS containing 0.1% BSA and 0.1% NaN_3 and finally re-suspended in PBS. Flow cytometric acquisition and analysis were performed with a FACS Calibur flow cytometer using the CellQuest software (BD Biosciences). The anti-CD3-induced phosphorylation increase was calculated by dividing the SLP-76 pY128 MFI values measured in activated cells with the MFI value of the resting cells.

Statistical analysis

Descriptive statistics was used to determine group means and the standard errors of the means (mean ± SEM). Differences between two groups were tested for statistical significance using Student's *t*-test. A value of $P \leqslant 0.05$ was considered statistically significant.

Results

Establishment of Jurkat cell lines transfected with ZAP-70 containing targeted point mutations at different tyrosine residues

To elucidate the role of different Y residues in TCR signaling, we have generated Jurkat cell lines stably expressing Y-F 'loss of function' point mutants of the ZAP-70 protein. To this end, we applied site-directed mutagenesis on the WT-ZAP-70 coding sequence to change Y residues 069, 126, 178, 238, 292, 315, 492 and 493 to F (Fig. 1A) leading to potential changes in signaling cascades based on the mass spectrographic data by Watts *et al.* (23). To date, no *in vivo* functional data were available about the first four (069, 126, 178 and 238) Y residues. The latter four residues are better known: Y292 and Y492 have been shown to possess inhibitory, whereas Y315 and Y493 possess activator role. This experimental setup was beneficial in two ways: (i) the known inhibitory and activator Y residues provided us internal controls to compare the effect of Y-F mutations of yet unknown residues on TCR signaling and (ii) we could validate/compare our data with those in previous works by other research groups. Importantly, this is the first systematic study in which eight targeted mutations in human ZAP-70 have been analyzed parallel using a knock-in cell line model.

The Y-F point mutant ZAP-70 constructs were introduced into P116 cells (ZAP-70-deficient Jurkat cells) using lentiviral transfection. The expression of the ZAP-70 molecule was confirmed with WB and flow cytometry (Fig. 1B and C). All transgenic cell lines expressed comparable levels of the ZAP-70 except the F238-ZAP-70 cell line (Fig. 1B and C).

The antibodies used for flow cytometry recognized two different parts of ZAP-70 (Fig. 1A). Importantly, the cell line expressing F292-ZAP-70 could not be stained with anti-ZAP-70-PE clone 1E7.2 because the epitope of this antibody lies between AAs 284 and 307. Therefore, targeted Y-F mutation at position 292 abrupted the epitope necessary for antigen recognition, which, in turn, verified the presence of this mutation (besides sequencing). The expression level of the ZAP-70 proved to be stable over time as checked regularly by intracellular ZAP-70 staining and flow cytometry (data not shown).

Interestingly, despite three repeated transfections, we could not establish the F238-ZAP-70-expressing cell line (Fig. 1B and C) although the construct was complete as assessed by repeated sequence and restriction analysis. As a consequence of this unsuccessful transfection, we omitted the analysis of Y238 from further parts of this study, despite the fact that Y238 is found in a putative immunoreceptor tyrosine-based inhibitory motif sequence (^{236}VEYLKL^{241}) based on an own preliminary sequence analysis (Fig. 1A) so it would have represented an interesting signaling target Y residue. Although we have no direct evidence why the expression of the F238-ZAP-70 was not successful, we might speculate that this mutation would affect post-translational modifications of the molecule leading to defective protein folding and subsequent breakdown.

Targeted Y-F point mutations in ZAP-70 alter the anti-CD3 treatment-induced Ca^{2+} signaling in Jurkat cells

Next, we checked if the transgenic ZAP-70 molecule was functionally active and restored the TCR/CD3 signaling pathway in P116 cells. In the absence of ZAP-70, the TCR signaling is blocked at an early stage leading to impaired T-cell activation *in vitro* and abnormal T-cell differentiation *in vivo* (44, 45). A plausible and simple way to check TCR/CD3 signaling is the analysis of anti-CD3-evoked Ca^{2+} signal. Here, confirming a previous study, introduction of the WT-ZAP-70 into P116 cells led to the restoration of the impaired anti-CD3-induced Ca^{2+} signal (Fig. 2) (46). Thus, the lentiviral trasfection led to the stable expression of a functional ZAP-70 molecule.

Targeted Y-F mutations had diverse effects on the anti-CD3-elicited Ca^{2+} signal (Fig. 2). As expected, mutation of the known inhibitory Y residues 292 and 492 led to an increased Ca^{2+} signal when compared with the WT-ZAP-70-expressing cells, while the mutation of the activator Y493 in the kinase domain completely abolished the anti-CD3-induced Ca^{2+} signal showing that the phosphorylation of this Y is essential for downstream TCR/CD3 signaling events (Fig. 2). Importantly, the opposing regulatory function of the neighboring Y492 and Y493 was mirrored in the Ca^{2+} signaling results (Fig. 2) and provided further proof that our site-directed mutagenesis approach was indeed precise. Mutation of the regulatory/activator Y315 led to a slightly decreased Ca^{2+} signal (Fig. 2).

The amplitude of the Ca^{2+} signal was significantly higher in the F069-ZAP-70-expressing cell line than in the WT-ZAP-70-expressing cell line, similar to what was found in the F292 and F492 mutant cell lines, indicating that Y069 could have an inhibitory role in TCR/CD3 signaling. Mutation at Y126 led to a significantly decreased Ca^{2+} signal, very similar to the effect of Y315 mutation. Based on this analogy, we propose that Y126 in interdomain A could have an activator/regulatory function-like Y315 has in interdomain B. Y-F mutation at the 178 AA position did not affect the anti-CD3-induced Ca^{2+} signaling.

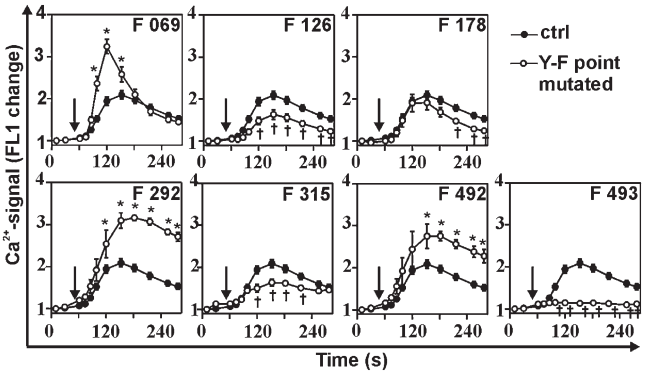

Fig. 2. Effect of Y-F mutations on the anti-CD3-elicited Ca2+ signaling in Jurkat cells. The Ca2+ signal was detected by flow cytometry after loading the cells with the Fluo-3AM indicator. Mean FL-1 fluorescence intensities (proportional with intracellular free Ca2+ level; y-axis) were plotted against time (x-axis). The effects of different point mutations were compared with the Ca2+ signal of the WT-ZAP-70-expressing cell lines (control); significantly ($P \leq 0.05$) higher (*) or lower (†) values are indicated. Data points in the diagrams represent mean ± SEM values calculated from three independent measurements. Ca2+ signals of the Y-F point mutants or the control are shown with empty or black dots, respectively. Anti-CD3 was added to the samples at 50 s (labeled with vertical black arrows) and the measurement continued for another 4 min.

To exclude the possibility that the transfections have changed the CD3 expression of the mutant cell lines and this would have led to the above differences in Ca^{2+} signaling, we checked the cell surface CD3 on all cell lines regularly but found no significant difference (data not shown).

Point mutations of specific Y residues in ZAP-70 alter tyrosine phosphorylation pattern and ZAP-70 autophosphorylation during TCR/CD3 signaling

First, we analyzed the anti-CD3 treatment-induced tyrosine phosphorylation patterns of the cell lines (Fig. 3A). Various Y-F mutations in the ZAP-70 molecule affected significantly the tyrosine phosphorylation of the cells. Phospho-protein bands of ~35, 55, 70 (most likely ZAP-70) and 115 kDa showed differences in the mutants compared with the WT-ZAP-70-expressing cells (Fig. 3A). A general hyperphosphorylation could be observed in the F292-ZAP-70-expressing cells (Fig. 3A). As expected, P116 ZAP-70-deficient cells did not activate upon anti-CD3 treatment (Fig. 3A). Densitometry of the 70-kDa band revealed that mutations at Y292 and Y493 led to a marked hyperphosphorylation (actin-corrected relative densities: 1.2 and 1.4, respectively) (Fig. 3A).

To confirm that the 70-kDa band observed on the whole cell lysate phospho-blot (Fig. 3A) was indeed ZAP-70, we performed IP with anti-ZAP-70 antibody (Fig. 3B, upper panels). We found increased phosphorylation of the ZAP-70 in the F292- and F493-ZAP-70 mutant cells (Fig. 3B, upper panels), which was in line with the results of the cell lysates (Fig. 3A). Y-F mutation at residue 315 led to slightly

decreased ZAP-70 phosphorylation (interestingly not seen in the cell lysates, Fig. 3A), while other mutations did not alter the anti-CD3-induced ZAP-70 phosphorylation (Fig. 3B, upper panels). Importantly, these results indicated that Y-F mutations at specific residues in ZAP-70 had an impact on the autophosphorylation (autoregulation) of the kinase.

SLP-76 phosphorylation is regulated by specific Y residues in ZAP-70

SLP-76 and LAT are two important substrates of ZAP-70 and their phosphorylation is critical for the activation of PLCγ and thus the initiation of Ca^{2+}-signaling (9, 11, 12). Therefore, next, we analyzed the phosphorylation of these two proteins using IP and WB. We found slight SLP-76 hyperphosphorylation in the F069-, F178-, F292-, F315- and F492-ZAP-70-expressing cell lines compared with the control WT-ZAP-70-expressing cells (Fig. 3B, lower panels). SLP-76 phosphorylation was impaired in the F493-ZAP-70 cells similar to P116 cells (Fig. 3B, lower panels), indicating that this activating residue is not only critical in the Ca^{2+} signal but also in phosphorylating SLP-76. Opposite to this, in case of LAT, Y-F point mutations in ZAP-70 did not alter the anti-CD3-induced phosphorylation significantly (data not shown). As expected, in P116 cells, phosphorylation of both SLP-76 and LAT was diminished due to the absence of ZAP-70.

With the Phospho-flow technique, phosphorylation events can be analyzed in a Y-specific manner. So next, we tested the above described phosphorylation changes in SLP-76 with the commercially available phospho-specific antibody recognizing the phosphorylation of Y128 (Fig. 4). We found a significantly decreased phosphorylation of SLP-76 Y128 in the anti-CD3-stimulated F493-ZAP-70-expressing cell line when compared with the WT-ZAP-70-transfected P116 cells (Fig. 4A and B) which was in line with the above IP experiment, indicating that Y128 in SLP-76 is phosphorylated by

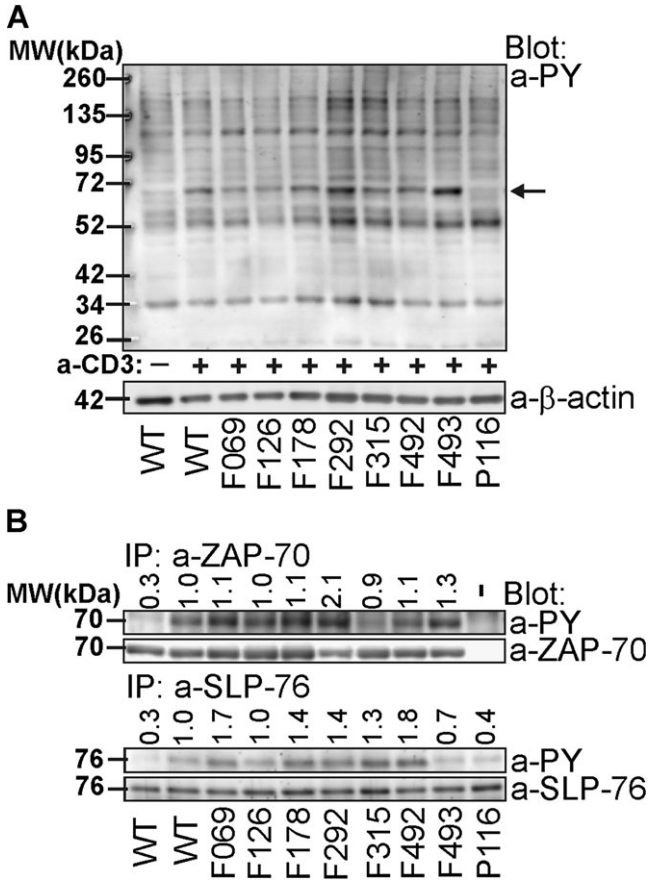

Fig. 3. Anti-CD3-induced phosphorylation in the Y-F mutant ZAP-70-expressing cell lines. (A): Whole cell lysates were separated on 10% SDS–PAGE and after blotting visualized with anti-phosphotyrosine antibody (upper panel). Arrow indicates the ZAP-70 band. Equal sample loading was verified by reprobing the blot with anti-β-actin antibody (lower panel). (B): The effect of ZAP-70 Y-F mutations on the Y-phosphorylation of ZAP-70 (upper panels) and SLP-76 (lower panels) was analyzed after IP. Blots were first probed with anti-phosphotyrosine antibody and then re-probed with anti-ZAP-70 or anti-SLP-76. Numbers above blot panels indicate the relative phosphorylation of the samples compared with the anti-CD3-activated control.

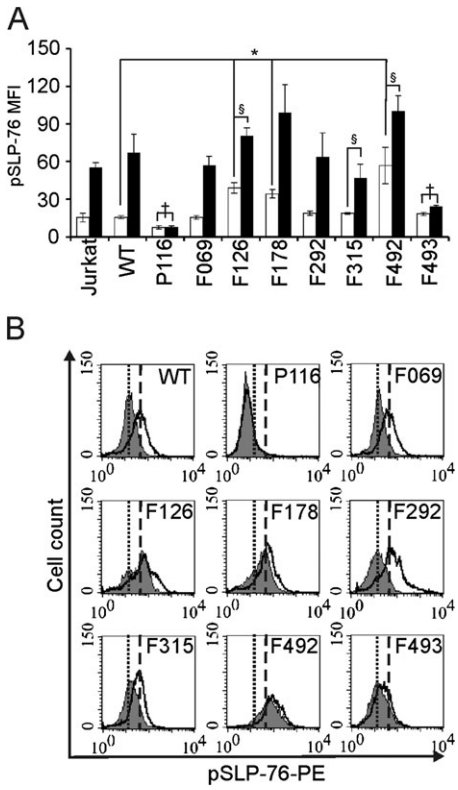

Fig. 4. Analysis of Y-specific SLP-76 phosphorylation changes with Phospho-flow technique in the WT or Y-F point mutant ZAP-70-expressing P116 cells. (A): Phosphorylation of SLP-76 Y128 in resting (white bars) or activated (black bars) cells is presented as pSLP-76 MFI. Bars show mean ± SEM calculated from the MFI data of three independent experiments. Significantly ($P \leq 0.05$) higher values compared with the 'WT' are indicated (*). The anti-CD3-induced SLP-76 Y128 phosphorylation was completely missing in P116 and F493-ZAP-70 cells (†) and markedly reduced in the F126-, F315- and F492-ZAP-70-expressing cell lines (§). (B): Corresponding representative flow cytometric histogram plots show the basal (gray filled histogram) and anti-CD3-activated (black histograms) pSLP-76 staining of the different cell lines. For easier comparison, MFI values of the basal and activated WT-ZAP-70-expressing (control) cell line are indicated with vertical dotted and dashed lines on all panels, respectively.

ZAP-70 in a Y493-dependent fashion. There was also a marked decrease of anti-CD3-induced Y128 phosphorylation in the F126-, F315- and F492-ZAP-70 cell lines (MFI change: 2.1 ± 0.1, 2.5 ± 0.6 and 2.0 ± 0.5, respectively) compared with the WT-ZAP-70-expressing cells (MFI change: 4.5 ± 1.31) (Fig. 4A and B). Interestingly, Y-F mutation at residues 126, 178 and 492 led to the hyperphosphorylation of Y128 of SLP-76 in unstimulated cells (Fig. 4A and B), indicating that these Y residues in ZAP-70 might possess autoinhibitory function.

Discussion

The TCR/CD3 signaling pathway has been extensively characterized in the last 20 years. During this time, there has been a significant shift from the initial theoretical, basic immunological and cell-signaling question toward the unraveling of its potential role in some immunological diseases and more, it became a promising therapeutic target. After the identification of the most important molecules of the TCR/CD3 signaling cascade, the use of mutant T-cell line (Jurkat) derivatives, defective in any of these signaling components, became a very valuable experimental validation process; for example, cell lines defective in the expression of Lck (JCaM1), ZAP-70 (P116), LAT (JCaM2.5) or SLP-76 (J14) have been widely utilized.

ZAP-70 is required for a complete TCR/CD3 signaling as shown by serious functional defects in ZAP-70-deficient cell line (P116) or mice (44, 45); moreover, in humans, defective ZAP-70 function leads to SCID phenotype (47). The function of kinases is regulated through phosphorylation events, therefore, studying the role of their potential phosphorylation sites is of particular interest and these studies can provide fine details of already known signaling pathways. In case of ZAP-70, point mutation studies are of special importance since some of these fine genetic changes lead to pathologic conditions, for example rheumatoid arthritis like disease in the SKG mice (48).

The role of Y292, Y315, Y492 and Y493 in ZAP-70 has been already characterized in previous studies (22); therefore, we included these Y residues into the present study as controls. Y292 and Y492 possess inhibitory role in TCR/CD3 signaling, while Y315 and Y493 are activator residues (24, 32, 49, 50). First, our Ca^{2+} signaling measurements were clearly in line with these data: mutations of either Y292 or Y492 to F led to increased Ca^{2+} signal amplitude, whereas mutations at Y315 or Y493 inhibited the anti-CD3-elicited Ca^{2+} signal partially or completely, respectively. Importantly, these four mutants exhibited various levels of positive or negative alterations in TCR/CD3 signaling, so, they provided us a basis for a correct comparison of the yet unknown Y residues.

Interestingly, relatively little is known about the function of Y residues found in the SH2 domains or interdomain A of ZAP-70 to date. Despite the fact that in the pioneer mass spectroscopy study of Watts *et al.* in 1994, three Y residues were identified in this region of ZAP-70; too, no further studies were performed either in cell lines or in mice to elucidate the potential regulatory role of these sites. Therefore, the point mutation analysis of Y069, Y126, Y178 and Y238 was of particular interest and importance since this might be the first systematic study where their potential regulatory role in the TCR/CD3 signaling pathway was investigated in detail. Although in the study of Watts *et al.* Y069, Y126 and Y178 were not phosphorylated upon anti-CD3 treatment, they showed that these residues might be phosphorylated by Lck *in vitro*; moreover, Y126 in interdomain A was found to be an autophosphorylation site as well (23).

Here, we found Y-F mutations at positions 069 and 126 had opposing effects on the Ca^{2+} signal: the former increased while the latter decreased the amplitude (Fig. 2, Table 1). Thus, these two Y residues seem to play a role in regulating TCR/CD3 signaling by ZAP-70. Y-F mutation at residue 178, on the other hand, did not alter the Ca^{2+} signal (Fig. 2, Table 1). Interestingly, Y-F mutation at residue 069 also altered the kinetics of the Ca^{2+} signal. Since Y069 is found within the N-terminal SH2 domain of ZAP-70, therefore, its effect on TCR/CD3 signaling might be mediated either through altered binding capacity of the activated ZAP-70 to the CD3 ζ chains (structural explanation) or, alternatively, this residue might impact the kinase activity of the molecule (autoregulatory/functional explanation). However, this question should be addressed in future studies.

Based on our results, Y126 in interdomain A might be a promising candidate in the regulation of ZAP-70 function and tuning of TCR/CD3 signaling. Loss of function mutation of Y126 led to a decreased Ca^{2+} signal, which was very similar to what was observed in case of Y-F mutation at residue Y315 (Fig. 2, Table 1). Based on this analogy, we propose that

Table 1. Summary of the changes in the studied signaling events caused by targeted mutagenesis of selected ZAP-70 tyrosines

Y-F position	Ca^{2+}	ZAP-70[a]	SLP-76[a]	SLP-76^{Y128}		Function	
a-CD3	+	+	+	-	+	TCR/CD3	Autoregulation
069	↑	—	↑	—	—	Inhibitory	None
126	↓	—	—	↑	↓	Activator	None
178	—	—	↑	↑	—	Part. inhibitory	None
238	NE	NE	NE	NE	NE	?	?
292	↑	↑	↑	—	—	Inhibitory	Negative
315	↓	↓	↑	—	↓	Activator	Positive
492	↑	—	↑	↑	↓	Inhibitory	None
493	↓	↑	↓	—	↓	Activator	Negative

All changes in signaling events were compared with the WT-ZAP-70-transfected (control) cell line. Y residues with previously undefined functions are indicated with italics. ↑, increase; ↓, decrease; —, no change; NE, not evaluated; part., partial.
[a]IP.

Y126 in interdomain A could have a similar positive regulatory role in ZAP-70-mediated signaling to Y315 in interdomain B (Table 1). Interestingly, mutation of Y126 in the ZAP-70 led to basal hyperphosphorylation of Y128 in SLP-76 (Table 1). Altered phosphorylation of this adapter molecule could contribute to the decreased anti-CD3-elicited Ca^{2+} signal.

An important implication of our present work was that the effect of specific Y-F point mutations in ZAP-70 on the anti-CD3-induced Ca^{2+} signal seemed to be mediated differentially by SLP-76 or LAT. Phosphorylation and activation of PLCγ are indispensable in T-cell Ca^{2+} signaling; the adaptor proteins SLP-76 and LAT are key activators of PLCγ, and they link ZAP-70 with PLCγ (9, 11, 12). Earlier data showed that regulation of PLCγ activation depends both on LAT and SLP-76 activation (51). In the present study, we could dissect these parallel pathways in more detail. Most Y-F mutations in ZAP-70 led to increased SLP-76 phosphorylation except at the important activator residue 493, which, not surprisingly, caused a decrease, and Y126, which had no effect (Fig. 3B, Table 1). Moreover, we identified that Y126, Y178 and Y492 in ZAP-70 regulate the basal phosphorylation of Y128 in SLP-76 (Fig. 4, Table 1). Phosphorylation of Y128 in SLP-76 recruits non-catalytic region of tyrosine kinase adaptor protein 1, P21/Cdc42/Rac1-activated kinase 1 and Wiskott-Aldrich syndrome protein to the activation complex caused by TCR/CD3 stimulation leading to cytoskeleton rearrangement (52). This way ZAP-70 might, through SLP-76, indirectly be involved in cytoskeleton regulation processes, too. Since LAT phosphorylation was not influenced markedly by ZAP-70 point mutations (data not shown), we propose that the fine regulation of ZAP-70 on the Ca^{2+} signal is preferentially mediated through SLP-76 not LAT, as indicated by the fine phosphorylation changes correlating more closely with the Y-F mutations in ZAP-70.

Finally, mutation of Y493 to F led to a serious block in all early signaling steps characterized in the present study to a similar extent to what was observed in the ZAP-70-deficient P116 cells. Therefore, phosphorylation of Y493 is critical in downstream steps of T-cell activation; its loss leads to a 'functional knock-down' state of the TCR/CD3 signaling pathway (Table 1), confirming earlier studies (24, 50). On the other hand, hyperphosphorylation of ZAP-70 could be observed in F493-ZAP-70-expressing cells, most likely due to the hyperphosphorylation of other Y residue(s). This indicates that Y493 phosphorylation has important negative autoregulatory function, too (Table 1); however, this question should be addressed in future experiments.

Our results indicated that Y493 is not the only residue, which might be involved in the autoregulation of ZAP-70. In our opinion, autophosphorylation of the ZAP-70 upon anti-CD3 treatment provides us information about the potential autoregulatory function of Y residues in the kinase: increased phosphorylation of the ZAP-70 in the point-mutated cell lines suggests a negative, while decreased phosphorylation a positive autoregulatory function of the respective tyrosine residue. Accordingly, we found that Y292 had negative autoregulatory role, besides Y493 discussed above: in their absence ZAP-70 was hyperphosphorylated. On the other hand, decreased ZAP-70 phosphorylation was detected in cells expressing F at residue 315, indicating its positive autoregulatory function (Fig. 3B, Table 1). These data complement previous results of Watts *et al.* about the *in vitro* autophosphorylation of Y126 and Y292 (23) and others suggesting that Y315 and 319 could posses positive autoregulatory function or participate in an 'autoinhibitory switch' regulating thereby the catalytic activity of ZAP-70 (22, 53). However, we are still far from the complete understanding of the fine mechanism and importance of the autoregulation/autophosphorylation of the ZAP-70 kinase.

In conclusion, TCR/CD3 signal transduction is influenced by a fine interplay of signaling molecules and ZAP-70 kinase with its Y residues conducts these early signaling events as summarized in Table 1. Our work confirmed the (auto)regulatory role of Y069, Y126 and Y178 residues of ZAP-70 in TCR/CD3 signaling completing our knowledge about specific phosphorylation sites found in this kinase molecule. Understanding fine details of ZAP-70 tyrosines in T-cell activation could bring us closer to unraveling its pathologic role in leukemia or autoimmune diseases, as well as future drug targeting.

Supplementary data

Supplementary data are available at *International Immunology* Online

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