Kinetic Analysis of ZAP70 Phosphorylation in Cell Lines and Human PBMCs: p-Y292, p-Y319, and p-Y493

David M. Soper, Efthalia Chronopoulou, Danny S. Madeux, Jeanne Elia, Xifeng Yang
BioLegend, San Diego, CA 92121

Abstract

The 70-kDa zeta chain-associated protein (ZAP70, Zap-70) is a Syk family protein tyrosine kinase normally expressed in T and NK cells. Patients deficient in ZAP70 have no functional T cells in the periphery, are prone to repeated infections, and suffer from ZAP70-related severe combined immunodeficiency (SCID). In some cases of chronic lymphocytic leukemia (CLL), ZAP70 is aberrantly expressed in B cells and used as a prognostic marker to monitor disease progression and therapeutic intervention. In normal T cells, ZAP70 is required for thymic development, T cell receptor (TCR) mediated signaling, and T cell activation. ZAP70 consists of two tandem Src homology 2 (SH2) domains, two linker regions, and a kinase domain. Following TCR engagement, several tyrosine residues within ZAP70 are phosphorylated and serve to both positively and negatively regulate kinase activity. BioLegend-developed clones were used to assess the phosphorylation status of three distinct ZAP70 sites in response to hydrogen peroxide (H_2O_2): anti-ZAP70 Phospho (Tyr319) / SYK Phospho (Tyr352), anti-ZAP70 Phospho (Tyr493), and anti-ZAP70 Phospho (Tyr292). A kinetic analysis (0, 5, 10, or 15 minutes) was performed on Jurkat cells and human peripheral blood mononuclear cells (PMBCs). In Jurkat cells treated with H_2O_2, ZAP70 phosphorylation is maintained throughout the time course analyzed. In human PBMCs, the kinetic responses vary among lymphocyte subsets: T cells ($CD3^+$) and NK cells ($CD3^-CD20^-$).

Methods

PBMC Isolation

Human PBMCs from healthy volunteers were isolated using Ficoll-Paque (GE Healthcare). Cells were then washed in phosphate buffered saline (PBS) and resuspended in DMEM with 1% FBS at a density of 10 x 10^6 cells/mL.

Cell Lines

The human T cell leukemic Jurkat cell line was grown in DMEM with 10% fetal bovine serum (FBS). Prior to hydrogen peroxide treatment, cells were cultured in DMEM with 0.01% FBS for 14-16 hours.

Hydrogen Peroxide Treatment

Prior to hydrogen peroxide treatment, Jurkat cells or human PBMCs were resuspended at 3-10 x 10^6 cells/mL and rested at 37°C for one hour. After resting, cells were treated with either media or hydrogen peroxide (5 mM for Jurkat cells and 2.5 mM for Human PBMCs, final concentrations) for 0, 5, 10, or 15 minutes.

Flow Cytometry Fixation/Permeabilization

Cells (PBMCs and cell lines) were fixed with Fixation Buffer (Cat. No. 420801) and permeabilized with True-Phos™ Perm Buffer (Cat. No. 425401).

Surface Staining

After fixation and permeabilization, human PBMCs were stained with Brilliant Violet 421™ anti-human CD3 (Cat. No. 300434) and PE/Cy7 anti-human CD20-cytoplasmic (Cat. No. 340514).

Intracellular Staining

After fixation and permeabilization, Jurkat cells and human PBMCs were stained Alexa Fluor® 488 anti-ZAP70 Phospho (Tyr319) / SYK Phospho (Tyr352), PE anti-ZAP70 Phospho (Tyr493), and Alexa Fluor® 647 anti-ZAP70 Phospho (Tyr292).

Instruments

Flow cytometry data was collected on a BD FACSCanto™ II

Lysate preparation

Following treatment with or without H_2O_2, cells were lysed with boiling lysis buffer containing Tris, SDS and sodium orthovanadate. To reduce sample viscosity, lysates were then sonicated and the protein concentration of the samples was determined by BCA assay.

Gel Electrophoresis and Protein Transfer

15 µg of total protein per lane was loaded in 4-12% Bis-Tris gels. Proteins were transferred to PVDF membrane using a wet transfer system (XCell SureLock™ mini cell system, Thermo Fisher).

Western blotting

PVDF membranes were blocked with 5% milk and blotted with purified antibodies specific for ZAP70 (Y319), ZAP70(Y292) and ZAP70(Y493) for 1 hr at 37°C. Blots were washed with TBS-Tween (TBST) and incubated with goat anti-mouse -HRP for 30 min at 37°C. Following washing with TBST, blots were developed with SuperSignal™ West Pico (Thermo Scientific) chemiluminescent substrate and exposed to X-ray film.

Figure 1: ZAP70 is rapidly phosphorylated and maintained in Jurkat cells

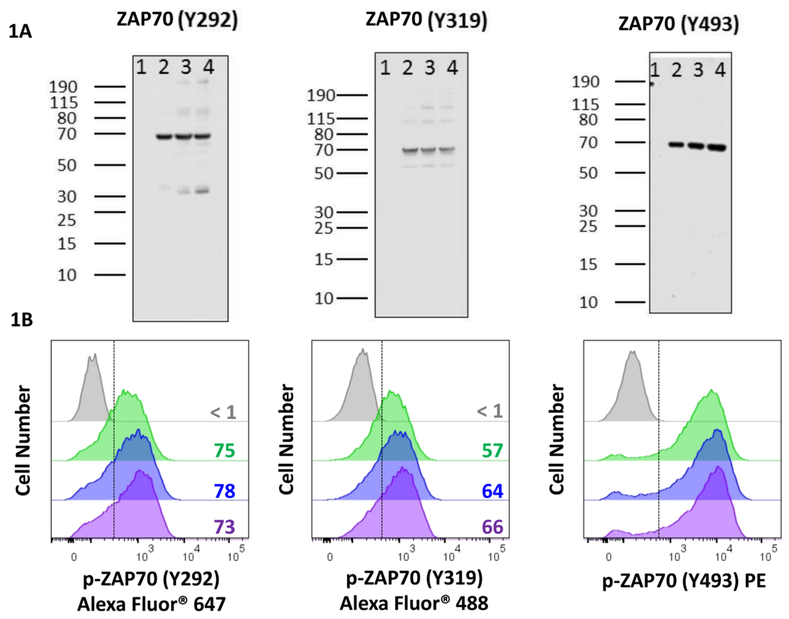

Figure 1: ZAP70 is rapidly phosphorylated and maintained in Jurkat cells. [Figure 1A] Blot of whole cell lysates derived from cells stimulated with 5 mM H_2O_2 for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3) and 15 min (lane 4). The levels of expression of phosphorylated ZAP70 (Tyr 493), ZAP70 (Tyr 319) and ZAP70 (Tyr 292) are indicated at about 70 kDa. [Figure 1B] Jurkat cells were treated with or without hydrogen peroxide for 0 (grey), 5 (green), 10 (blue), or 15 (purple) minutes, fixed with Fixation Buffer, permeabilized with True-Phos™ Perm Buffer, and then intracellularly stained with Alexa Fluor® 488 anti-ZAP70 Phospho (Tyr319) / SYK Phospho (Tyr352), PE anti-ZAP70 Phospho (Tyr493), and Alexa Fluor® 647 anti-ZAP70 Phospho (Tyr292). The values in each graph indicate the percentage of cells beyond the no stimulation control (as indicated by the dotted line).

Legend
No Stimulation
5 min
10 min
15 min

Figure 2: Human PBMC lymphocyte subsets exhibit differential ZAP70 phosphorylation kinetics

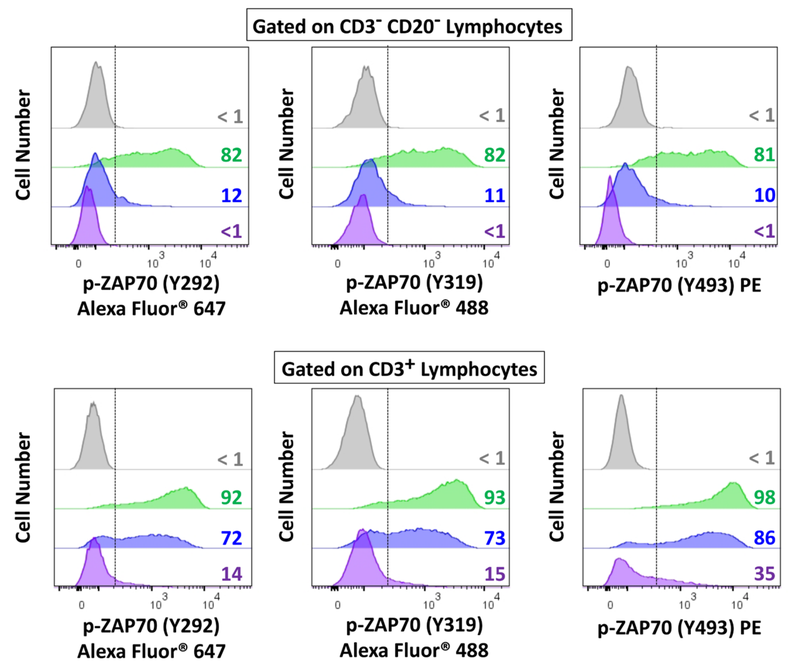

Figure 2. Human PBMC lymphocyte subsets exhibit differential ZAP70 phosphorylation kinetics. Human peripheral blood lymphocytes were treated with or without hydrogen peroxide for 0 (grey), 5 (green), 10 (blue), or 15 (purple) minutes, fixed with Fixation Buffer, permeabilized with True-Phos™ Perm Buffer, and then stained with Brilliant Violet 421™ anti-human CD3 (Cat. No. 300434), PE/Cy7 anti-human CD20-cytoplasmic (Cat. No. 340514), Alexa Fluor® 488 anti-ZAP70 Phospho (Tyr319) / SYK Phospho (Tyr352), PE anti-ZAP70 Phospho (Tyr493), and Alexa Fluor® 647 anti-ZAP70 Phospho (Tyr292). The values in each graph indicate the percentage of cells beyond the no stimulation control (as indicated by the dotted line). Data from one donor is shown but is representative of 5 donors analyzed.

Legend
No Stimulation
5 min
10 min
15 min

Results

In-house clones were developed and used to assess the phosphorylation status of three distinct ZAP70 sites in response to H_2O_2 stimulation: anti-ZAP70 Phospho (Tyr319) / SYK Phospho (Tyr352), anti-ZAP70 Phospho (Tyr493), and anti-ZAP70 Phospho (Tyr292). In Jurkat cells, the data indicate H_2O_2 induces ZAP70 phosphorylation, which is sustained for up to 15 minutes. In human PBMC lymphocytes, we observed that NK and T cells exhibit differential phosphorylation kinetics.

Conclusions

The tyrosine kinase ZAP70 is required for the normal development of T cells. Our data indicate that cell lineage plays an important role in both the kinetics and the magnitude of H_2O_2 induced phosphorylation. Future experiments using physiological stimulations (NKG2D or CD3/CD28 cross-linking) will expand our understanding of cell type-specific signaling and how differential regulation of signaling impacts on immune function. These antibodies are tools to study aberrant expression of ZAP70 in disease states (*e.g.,* CLL).

04-0083-00