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Modification by covalent reaction or oxidation of cysteine residues in the Tandem-SH2 Domains of ZAP-70 and Syk Can Block Phosphopeptide Binding

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Abstract

Zeta-chain Associated Protein of 70kDa (ZAP-70) and Spleen tyrosine kinase (Syk) are non-receptor tyrosine kinases that are essential for T-cell and B-cell antigen receptor signaling, respectively. They are recruited, via their tandem-SH2 domains, to doubly-phosphorylated Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) on invariant chains of immune antigen receptors. Because of their critical roles in immune signaling, ZAP-70 and Syk are targets for the development of drugs for autoimmune diseases. We show that three thiol-reactive small molecules can prevent the tandem-SH2 domains of ZAP-70 and Syk from binding to phosphorylated ITAMs. We identify a specific cysteine residue in the phosphotyrosine-binding pocket of each protein (Cys 39 in ZAP-70, Cys 206 in Syk) that is necessary for inhibition by two of these compounds. We also find that ITAM binding to ZAP-70 and Syk is sensitive to the presence of hydrogen peroxide, and these two cysteine residues are also necessary for inhibition by hydrogen peroxide. Our findings suggest a mechanism by which the generation of reactive oxygen species generated during responses to antigen could attenuate signaling through these

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kinases, and may also inform the development of ZAP-70 and Syk inhibitors that bind covalently to their SH2 domains.

Keywords

hydrogen peroxide; covalent small molecule inhibitor; T-cell; B-cell; ITAM

Introduction

Zeta-chain associated protein of 70-kDa (ZAP-70) and Spleen tyrosine kinase (Syk) are non-receptor tyrosine kinases that are essential for adaptive immune signaling [1, 2]. ZAP-70 plays a key role in bridging the stimulation of the T-cell antigen receptor with T-cell activation. Upon antigen binding to the T-cell antigen receptor, Lck, the membrane-bound and CD4/CD8 coreceptor associated tyrosine kinase, phosphorylates the intracellular ζ -chains of the CD3 coreceptor on Immunoreceptor Tyrosine Activation Motifs (ITAMs), which are defined by the sequence motif (D/E)xxYxx(I/L)x₍₆₋₈₎Yxx(I/L), where x represents a variable position. ZAP-70 is then recruited to the T-cell antigen receptor, where it is activated and colocalized to its substrates, Linker for activation of T-cells (LAT) and SH2-containing linker protein of 76-kDa (SLP-76). A similar signaling cascade occurs in B-cells, in which the non-receptor tyrosine kinase Syk interacts with ITAM motifs in the B-cell antigen receptor.

ZAP-70 and Syk share a common domain architecture and are composed of tandem-SH2 domains (an N-terminal or N-SH2 domain and a C-terminal or C-SH2 domain) connected by a coiled-coil linker (inter-SH2 linker or Interdomain A) and a kinase domain that is connected by a largely unstructured linker (SH2-kinase linker or Interdomain B) to the tandem-SH2 module (Figure 1) [3-6]. The tandem-SH2 modules (referred to as ZAP-tSH2 in ZAP-70 and Syk-tSH2 in Syk) recognize and bind to doubly-phosphorylated ITAMs. Engagement of the tandem-SH2 module with ITAMs is coupled to a conformational change in ZAP-70 and Syk that enables phosphorylation of the SH2-kinase-linker and release of autoinhibitory interactions [6].

T-cell antigen receptor signaling can be halted by receptor internalization [7], which can be accomplished through several different mechanisms [8, 9]. B-cell antigen receptor internalization occurs through similar mechanisms [10, 11]. The action of tyrosine kinases is counteracted by the activity of protein tyrosine phosphatases, but tandem-SH2 binding to ζ -chains has been shown to protect phosphorylated ITAMs from phosphatases [12]. Additionally, the formation of an immunological synapse is thought to exclude receptor-type protein tyrosine phosphatases [13, 14]. Whether ZAP-70 or Syk are inactivated directly after the initiation of immune cell signaling is not known, but the ongoing action of ZAP-70 is required to sustain signaling [15].

The importance of reactive oxygen species in cellular signaling has been characterized in a number of systems. Reactive oxygen species can oxidize cysteine residues, and this can play either activating or inhibitory roles in signaling proteins [16]. Hydrogen peroxide (H₂O₂) was first found to modulate tyrosine phosphorylation signaling through inhibition of protein

tyrosine phosphatases. H_2O_2 oxidizes the catalytic active site cysteine residues of these phosphatases, inactivating them and increasing the levels of tyrosine phosphorylation [17-19]. Later studies revealed that tyrosine kinases are also subject to oxidative regulation. Two Src-family kinases, c-Src and Lyn, are activated by sulfenylation [20, 21]. The activity of the epidermal growth factor receptor increases when a cysteine residue at the active site is oxidized [22].

The level of reactive oxygen species is thought to be controlled dynamically in response to the strengths of the signal. For example, the enzyme peroxiredoxin converts H_2O_2 to H_2O at low H_2O_2 levels, serving as a “floodgate” that maintains reactive oxygen species at a low level [23]. However, at higher concentrations of H_2O_2 , the peroxiredoxin is inactivated through oxidation of the catalytic cysteine residue. This opens the peroxide floodgate and allows for the propagation of H_2O_2 signaling. More recent work has shown that peroxiredoxins can also be inactivated through phosphorylation on a specific tyrosine residue [24]. This suggests that a microenvironment may exist near active kinases at the plasma membrane, within which H_2O_2 levels can be modulated rapidly.

The activation of T-cells has been shown to induce the rapid production of reactive oxygen species [25] through superoxide dismutases [26, 27] as well as NADPH oxidase [28]. The role of redox signaling as a modulator of T-cell activity is just beginning to be elucidated [29]. Reactive oxygen species also seem to play a similar role in the regulation of B-cells [30, 31]. However, little work has been done to determine the molecular basis of regulation by oxidation in lymphocyte signaling.

ZAP-70 contains nine cysteine residues in the tandem-SH2 module and seven cysteine residues in the kinase domain (Figure 1). Cys 39 in ZAP-70 is located in the phosphotyrosine-binding pocket of the N-terminal (N-SH2) domain. A corresponding cysteine residue is absent in Syk, but the phosphotyrosine binding pocket of the C-terminal (C-SH2) domain of Syk does contain Cys 206 (there is no corresponding cysteine residue in ZAP-70). Here we examine the effect of cysteine modification on the ability of the isolated tandem-SH2 modules of ZAP-70 and Syk to bind to an ITAM-derived peptide. In addition, small molecule inhibitors of ZAP-70 that were identified in a high throughput screen (to be published) mediate their inhibitory effects by reacting covalently with cysteine residues in both ZAP-70 and Syk.

Experimental

Molecular cloning and plasmid preparation

The tandem-SH2 modules of human ZAP-70 (residues 1-259 or 1-254) or human Syk (residues 6-269) were subcloned into pET28 plasmids (EMD Millipore) using NdeI and NotI restriction sites. A hexa-histidine tag, PreScission Protease (GE Healthcare Life Sciences) cleavage site, and Gly-Ser-Gly linker were inserted N-terminal to the ZAP-70 or Syk sequences. Site directed mutagenesis was performed with Quickchange (Stratagene).

Protein expression and purification

Various ZAP-70 and Syk tandem-SH2 constructs were expressed in *E. coli* BL21(DE3) cells in Terrific Broth at 37°C until they reached an OD = 1.0. Cells were then induced with 1 mM IPTG and grown overnight at 18°C. Cells were collected by centrifugation at 3600 × *g* (Sorval RC 3C Plus) for 10 minutes and resuspended in a buffer containing 50 mM Tris pH 8.0, 50 mM NaCl, 20 mM imidazole. Resuspended cells were then lysed by French press. This lysate was centrifuged for 30 minutes at 37000 × *g* (Sorvall Evoluton RC). His-tagged protein was purified from the supernatant using a Ni-NTA column (GE Healthcare Life Sciences) and eluted with buffer containing 500 mM imidazole. 2 mM DTT was added and the His-tag and the PreScission protease recognition site were removed by overnight cleavage with PreScission Protease at 4°C. The ZAP-tSH2 or Syk-tSH2 samples were then exchanged into a buffer containing 20 mM Tris pH 8.5, 150 mM NaCl. This material was then passed over a Ni-NTA column and a Q column (GE Healthcare Life Sciences), and the flowthrough collected. The protein was then run over an S200 gel filtration column (GE Healthcare Life Sciences) in buffer containing 20 mM Tris pH 8.0, 150 mM NaCl, 5% Glycerol, and 1 mM TCEP, and the protein was then concentrated to ~ 25 mg/mL by centrifugation in 3000 MW spin columns (EMD Millipore). Expression and purification of the ZAP-70 kinase domain (residues 327-606) has been previously reported [6].

Preparation of fluorophore-labeled ITAM-peptide

A peptide with sequence corresponding to that of the first ITAM motif of the T-cell antigen receptor ζ-chain (CGNQL(pY)NELNLGRREE(pY)DVLD; where pY is phosphotyrosine) including an N-terminal cysteine residue followed by a glycine linker (prepared by David King, UC Berkeley/HHMI) was resuspended in 20 mM Tris buffer and the pH was adjusted to 7.5. The peptide was reduced by incubation with 3-fold excess of TCEP and incubated at 50°C for 30 min. This peptide was then conjugated via the thiol group of the cysteine residue to a maleimide moiety on AlexaFluor488 (Life Technologies). Labeled peptides were purified by reverse phase chromatography on a C18 HPLC column using a water and acetonitrile gradient with 0.01% trifluoroacetic acid. Correct labeling was confirmed by mass spectrometry. We refer to this peptide as the 2pY peptide.

Thiol-reactive compounds

We describe the interaction of three small molecules, referred to as compounds **A**, **B**, and **C**, with ZAP-70 or Syk. These compounds were identified in a high throughput screen for ZAP-70 inhibitors; the detail of which will be published separately (Visperas et al. in preparation). The chemical structures of these compounds are shown in Figure 2. The compound suppliers are as follows: Compound **A** was obtained from InterBioScreen Ltd. (Supplier ID: STOCK5S-74623). Compound **B** was obtained from Pharmeks Ltd. (Supplier ID: PHAR025826). Compound **C** was obtained from ChemBridge Corp. (Supplier ID 7997218). The compounds were obtained in powdered form and dissolved at 50 mM in DMSO. The purity of all compounds was assessed and their correct masses verified by LC/MS. All compounds submitted for testing were judged to be of 95% or higher purity based on analytical LC-MS analysis on a Waters Micromass ZQ instrument, through UV absorbance at 254 nm and light scattering. Separations were carried out on an XTerra® MS

C18 5 μ m 4.6 $\times$ 50mm column at ambient temperature using a mobile phase of water-methanol containing 0.1% formic acid. Chemical identity was confirmed in positive ion mode by the presence of the anticipated molecular weight M+1 ion. No purification was performed on the compound solutions.

ZAP-70-tSH2:compound A crystallization, data collection, and structure determination

The tandem-SH2 module of ZAP-70 (wild type sequence, residues 1-259) at 20 mg/mL was preincubated with a 1.1-fold molar excess of compound **A** for 15 minutes before being spun down at 16,000 $\times$ g at 4°C for 10 minutes. 1 μ L of this tandem-SH2:compound **A** solution was mixed with 1 μ L of well solution (7.75% PEG 3350, 1.10 M ammonium sulfate, 5 mM TCEP) and equilibrated against the well solution in a hanging drop set up for 16 hours at 20°C. The crystals were soaked in a cryoprotectant composed of lithium acetate dissolved in well solution until lithium acetate would no longer dissolve. Crystals were then frozen in liquid nitrogen. Data collection was performed at the Advanced Light Source (Lawrence-Berkeley National Lab, Berkeley, CA) on beamline ALS8.2.1.

ZAP-70-tSH2:compound B crystallization, data collection, and structure determination

A variant of the ZAP-70 tSH2 module in which a cysteine residue was replaced with serine (residues 1-259, C117S) at 5 mg/mL was pre-incubated with a two-fold molar excess of compound **B** (see figure 2 for the chemical structures of the compounds used in this study) and a 1.5-fold molar excess of doubly-phosphorylated ITAM peptide for 15 minutes before being spun down at 16,000 $\times$ g at 4°C for 10 minutes. 1 μ L of this tandem-SH2:ITAM:compound **B** solution was mixed with 1 μ L of well solution (24% PEG 6000, 220 mM calcium chloride, 100 mM MES pH 6.0, and 20 mM TCEP) and equilibrated against the well solution in a hanging drop set up for 16 hours at 20°C. The crystals were soaked in a cryoprotectant of well solution with 30% glycerol and frozen in liquid nitrogen. Data collection was performed at the Advanced Light Source (Lawrence-Berkeley National Lab, Berkeley, CA) on beamline ALS8.3.1.

Protein alkylation

ZAP-tSH2 at 1 mg/mL ($\sim$ 30 μ M) was incubated with a ten-fold excess ($\sim$ 300 μ M) of iodoacetamide (Sigma-Aldrich) or n-ethylmaleimide (Sigma-Aldrich) and incubated at room temperature overnight. Excess alkylating compound was quenched with 1 mM DTT. Quenched compound and DTT were removed through at least 5 rounds of 1:20 dilution and concentration with 3000 MW spin concentrators (EMD Millipore) for a final dilution of 1:20⁵ or 1:3.2 $\times$ 10⁶.

Fluorescence polarization-based ITAM-binding assays

Fluorescence polarization assay buffer (150 mM NaCl, 20 mM Tris pH 8.0, 2% glycerol, 0.01% Triton X-100, 0.01% Tween-20), 1 mM TCEP, 100 nM ZAP-tSH2, and 2 nM AlexaFluor488-2pY were premixed in total volume of 80 μ L. Each experiment was begun with the addition of 1 μ M compound and a final concentration of 1% DMSO before transfer to a 384-well plate (Corning 3820). Fluorescence polarization was measured by using a

Biotech Synergy H4 fluorescence plate reader. Fluorescence polarization binding assays with Syk-tSH2 were performed similarly, but in the absence of TCEP.

Insect cell expression and purification of ZAP-70 constructs

A ZAP-70 construct containing a portion of the SH2-kinase linker and the ZAP-70 kinase domain (containing residues 310 to 619), a subsequent Gly-Ser-Gly linker, a PreScission protease site, and a C-terminal 6 $\times$ -His tag, was cloned into the pFastBac1 (Life Technologies) vector with BamHI and EcoRI restriction sites. This construct was expressed in baculovirus-infected TriEx Sf9 cells and purified as previously described [6].

Cloning of SMT3-LAT-Y7C and Grb2-SH2-C constructs

A LAT-derived peptide sequence containing residues 163-179 with an N-terminal Gly-Ser linker and a C-terminal Gly-Cys was cloned into a pET vector containing 6 $\times$ -histidine-tagged SMT3 using BamHI and XhoI restriction sites. Grb2-SH2 was cloned into a modified pET vector containing the Grb2-SH2 construct with an N-terminal His-tag and TEV protease cleavage site, and a C-terminal Cys (HisTEV-hGrb2-SH2-C).

Bacterial expression and purification of SMT3-LAT-Y7C

The LAT peptide construct (SMT3-LAT-Y7C) was expressed by transformation and growth in *E. coli* BL21(DE3) cells in Terrific broth (TB) at 37°C until they reached an OD = 0.5, and then the temperature was reduced to 18 °C. After 30 min, cells were then induced with 1 mM IPTG and grown overnight at 18°C. Cells were collected by spinning at 4000 rpm in a Sorval centrifuge for 10 minutes and resuspended in a buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 25 mM imidazole. The cells were then lysed using a French press and centrifuged at 42,000 rpm for 1 h at 4°C to pellet cellular debris. His-tagged protein was purified from cell lysate using a Ni-NTA column (GE Healthcare) and eluted with buffer containing 500 mM imidazole. The LAT peptide sample was then diluted with 50 mM Tris pH 8.0 into to a buffer containing 50 mM imidazole and 50 mM NaCl. This solution was flowed through a Q column (GE Healthcare), and the pure fractions containing desired protein (a band at ~17 kDa) were collected. The protein was then concentrated to 50 mg/mL by centrifugation in 3000 MW spin columns (Millipore).

Bacterial expression and purification of Grb2-SH2-C

The Grb-2SH2-C construct was expressed by transformation and growth in *E. coli* BL21(DE3) cells in Terrific broth at 37°C until they reached an OD = 1.5, and then diluted with an equal volume of cold TB. Cells were induced with 1 mM IPTG and grown overnight at 25°C. Cells were collected by spinning at 4000 rpm in a Sorval centrifuge for 15 minutes and resuspended in a buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 25 mM imidazole, 10% glycerol, and 10 mM MgCl₂, then lysed by disruption and centrifuged at 42,000 rpm for 1 h at 4°C. His-tagged protein was purified from cell lysate using a Ni-NTA column (GE Healthcare) and eluted with buffer containing 500 mM imidazole. The Grb2-SH2-C construct was dialyzed with 20 mM β -mercaptoethanol (BME) and TEV into buffer containing 50 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol, and ~20 mM BME overnight at 4°C. The cleaved protein was run over a subtractive Ni-NTA column and eluted with

buffer containing 500 mM imidazole. The protein was then run over a S75 gel filtration column (GE Healthcare) in buffer containing 25 mM TEA pH 7.5, 100 mM NaCl, and 1 mM TCEP and collected.

Preparation of fluorophore-labeled SMT3-LAT-Y7C and Grb2-SH2-C

Alexa Fluor 555 C₂ maleimide and Alexa Fluor 647 C₂ maleimide were purchased from Life Technologies. SMT3-LAT-Y7C was incubated in a 2-fold molar excess of Alexa Fluor 555 C₂ maleimide, and Grb2-SH2-C was incubated in a 2-fold molar excess of Alexa Fluor 647 C₂ maleimide, both for 2 h at room temperature. Free dyes were separated from protein by size exclusion chromatography (Sephadex G-25).

FRET-based kinase assay

The phosphorylation of a AlexaFluor 555-labeled SMT3-LAT by ZAP-70 was monitored by measuring the increase of AlexaFluor 647 fluorescence that accompanies the recruitment of AlexaFluor 647-labeled Grb2-SH2 to phosphorylated AlexaFluor 555-labeled SMT3-LAT. The data were normalized by dividing the acceptor fluorescence (AlexaFluor 647) by the donor fluorescence (AlexaFluor 555). This value represents an acceptor:donor ratio or FRET ratio. The reaction was performed in a buffer containing 20 mM Tris pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM TCEP, 1 mM NaVO₄, 5% Glycerol, 0.01% Tween 20, 20 μM ATP, 1 μM SMT3-LAT, 1 μM Grb2-SH2, and 50 nM of ZAP-70 kinase domain. The reaction was initiated by adding ATP to a final concentration of 20 μM and 100 μL total reaction volume. The reaction was transferred to a 384-well plate (Corning 3820). Measurements were taken every minute on a Biotek Synergy H4 fluorescence plate reader.

Results

Compounds that modify cysteine residues can inhibit binding of the doubly phosphorylated ITAM peptide to the tandem-SH2 module of ZAP-70

A high-throughput screen carried out at the UCSF Small Molecule Discovery Center resulted in the identification of several compounds that inhibited the binding of ZAP-70 to a doubly-phosphorylated ITAM-derived peptide (the 2pY peptide). The details of this screen will be published elsewhere [Visperas, et al. *in preparation*]. Here we show that three of these compounds (see Figure 2 for their chemical structures) react covalently with the tandem-SH2 modules of ZAP-70 or Syk, and that the modification of specific cysteine residues underlies the inhibition of 2pY binding (Figure 5).

Our first indication that these compounds react covalently with the tandem-SH2 module of ZAP-70 came from analysis of crystal structures. We obtained crystal structures for two of the compounds (compounds **A** and **B**) bound to the tandem-SH2 module of ZAP-70. In both cases the electron density showed clear evidence for covalent bond formation at one particular cysteine residue in each case. As discussed below, the cysteine residue that is modified is different in the two crystal structures, and in neither case does it appear that the particular cysteine modification that is visualized would block ITAM binding. Nevertheless, these structures alerted us to the possibility of covalent binding by these compounds, which prompted the investigation of the time-dependence of the inhibition, described later.

A co-crystal structure of ZAP-tSH2 with compound **A** determined at a resolution of 2.0 Å shows a single covalent adduct on Cys 117 (Figure 3). Although there is no ITAM present, the SH2 domains assume a conformation that resembles the ITAM-bound conformation [3] more closely than it does the conformation of the tandem-SH2 module in the absence of peptide [32]. In the structure of the complex with compound **A**, the peptide binding surfaces of the two SH2 domains are oriented relative to each other nearly as they are in the ITAM-bound complex, even though there is no ITAM bound [3]. In place of the phosphate groups of the ITAM, sulfate ions occupy the phosphotyrosine binding pockets.

The compound **A** adduct forms a π -stacking interaction with the sidechain of a tyrosine residue from a neighboring molecule in the crystal lattice. This interaction causes a rotation of Cys 117 that partially unwinds the interdomain A helix. The compound **A** adduct does not appear to make any interactions that would potentially interrupt ITAM binding, and so we conclude that adduct formation with Cys 117 is unlikely to explain the inhibition of ITAM-binding by compound **A**.

The visualization of this adduct prompted an analysis by mass spectrometry of the ZAP-tSH2 module after the addition of compound **A**. In this experiment, compound **A** was incubated with ZAP-tSH2 in a 2-fold molar excess for 16 hours, which mimicked the crystallization conditions. This analysis confirmed that compound **A** did in fact form covalent adducts with a mass increase of 186 Da (Figure 2). Prompted by this observation and by the crystal structure of ZAP-tSH2 with compound **A**, we replaced Cys 117 with serine. Attempts to crystallize ZAP-tSH2 C117S with compound **A** were unsuccessful. We were however, able to obtain a co-crystal structure of ZAP-tSH2 C117S with compound **B**.

A co-crystal structure of the ITAM-bound complex of ZAP-tSH2 with compound **B** determined at 2.9 Å resolution revealed a covalent adduct on Cys 78 (Figure 4). This structure is otherwise nearly identical to that of the ITAM-bound tandem-SH2 module reported previously [3]. The adduct formed on Cys 78 does not make any contacts within the protein or with any neighboring molecules in the crystal lattice. There is no structural indication of disruption of ITAM binding. The co-crystallization of compound **B** with ZAP-tSH2 C117S was carried out in a 2-fold excess of compound over protein so we presume that the structure simply reflects the higher reactivity of compound **B** with Cys 78 under the crystallization conditions, and that adduct formation at this residue does not block peptide binding. Mass spectrometry confirmed that compound **B** forms two covalent adducts with a mass increase of 222 Da with ZAP-tSH2 (Figure 2). We also found that a third compound, compound **C** forms a single adduct with a corresponding mass of 359 Da. This suggests that compound **C** may not exhibit the same degree of reactivity or promiscuity as compounds **A** or **B**.

That compounds **A**, **B**, and **C** inhibit ZAP-70 binding to the 2pY peptide by a process that involves covalent modification is suggested by a clear time-dependence in the extent of inhibition. When monitoring fluorescence polarization (FP) as a function of time in the presence of each of the compounds, there is a decrease in polarization that is indicative of ZAP-tSH2 and 2pY peptide dissociation (Figure 5). The DMSO control shows no decrease in polarization, and therefore, no change in binding. Free peptide is used as a positive

control of inhibition to determine a baseline fluorescence polarization value. Results are normalized to maximum and baseline fluorescence polarization values. Replacement of the phosphine-containing reductant TCEP with the thiol-containing reductant DTT abolished inhibition by all three of the compounds and further confirmed that the compounds are thiol-reactive (Supplement 1).

Of the nine cysteine residues in the tandem-SH2 module of ZAP-70, five (Cys 39, Cys 78, Cys 84, Cys 96, and Cys 102) are in the N-SH2 domain. Cys 117 is located in the inter-SH2 domain linker. Three others (Cys 222, Cys 249, and Cys 254) are found in the C-SH2 domain. Based on crystal structures of full-length ZAP-70 or the isolated tandem-SH2 domains, we hypothesized that Cys 39 is the residue that is crucial for compound inhibition. Cys 39 is located at the base of the phosphotyrosine binding pocket in the N-SH2 domain of ZAP-70. It is likely that covalent modification of this residue results in a steric obstruction that occludes phosphotyrosine binding. We found that the C39S mutation protects ZAP-tSH2 from inhibition by compounds **B** and **C**, but not compound **A** (Figure 5). We do not further characterize inhibition by compound **A**. Mutation of other cysteine residues in ZAP-tSH2 had no effect on compound inhibition of ZAP-tSH2 binding of 2pY (Supplement 2). Replacing Cys 39 with serine results in reduced compound labeling on ZAP-tSH2 as monitored by HPLC (Supplement 3) and mass spectrometry (Supplement 4 and summarized in Supplement 5).

ZAP-tSH2 is sensitive to oxidation and requires Cys 39 for inhibition

We typically use 1 mM TCEP to maintain reducing conditions in our binding assays. Under these conditions, we observe little to no change in polarization over the course of the experiment. However, upon decreasing the concentration of TCEP in our assays, we noticed a time- and concentration-dependent decrease in binding (Figure 5). This apparent dissociation of 2pY peptide occurs within fifteen minutes at TCEP concentrations ranging from ~60 μ M to 1 μ M. We presume that upon reducing TCEP concentrations, cysteine residues in the tandem-SH2 module oxidize spontaneously, which results in dissociation of the 2pY peptide. We went on to test this idea by adding hydrogen peroxide to directly oxidize the protein in the presence of TCEP. In both a time-dependent and concentration-dependent manner, we observed a decrease in 2pY peptide binding with the addition of hydrogen peroxide (Figure 5). This inhibition due to oxidation could be observed with the addition of as little as 1 μ M H₂O₂, which is nearly stoichiometric with the total amount of cysteine residues.

The apparent inhibition that we observed could arise from nonspecific effects of oxidation. For example, oxidizing conditions could allow ZAP-tSH2 to form intermolecular disulfide bonds, resulting in aggregation of the protein and dissociation of the peptide. Complete oxidation of the protein on cysteine and methionine residues could cause the protein to denature, releasing the peptide. However, the behavior of the C39S mutant suggests that the inhibition of the peptide binding by oxidizing conditions is not the result of nonspecific effects. We titrated down the concentration of TCEP to decrease the reducing conditions in the 2pY-binding assay with ZAP-tSH2 C39S. In contrast to what is seen for the wild type protein, the C39S mutant is not inhibited when the levels of TCEP are reduced, suggesting

that the inhibition of the wild-type protein is due to the specific oxidation of Cys 39 (Figure 5).

To directly test the effect of oxidizing conditions on 2pY binding of ZAP-tSH2 C39S, we titrated hydrogen peroxide into the binding reaction to increase the oxidative conditions of the assay. In contrast to the wild type protein, the C39S protein is not inhibited by hydrogen peroxide (Figure 5). Taken together, these experiments demonstrate that 2pY binding of ZAP-tSH2 is sensitive to oxidation. Furthermore, Cys 39 is necessary for this inhibition. We have not determined the oxidation state of Cys 39 that is correlated with inhibition, which will be the focus of future study. Nevertheless, we speculate that the oxidation of Cys 39 might introduce a negative charge or the additional bulk to the phosphotyrosine binding pocket; which might disfavor binding of the negatively-charged phosphotyrosine residue in the ITAM peptide.

Cysteine modification also inhibits the binding of doubly-phosphorylated ITAM to the tandem-SH2 module of Syk

ZAP-70 and Syk share a common domain architecture and have also been shown to bind various ITAM motifs with very similar affinities [33]. For these reasons, we wondered whether the tandem-SH2 unit of Syk could be subject to the same inhibition by cysteine modification as that of ZAP-70.

To test this, we measured the binding of the 2pY peptide to the tandem-SH2 module of Syk by monitoring fluorescence polarization. We found that Syk-tSH2 could be inhibited by compound **B** and compound **C** in a time-dependent manner (Figure 6). Inhibition occurred within thirty minutes for Syk-tSH2 as with ZAP-tSH2. Hydrogen peroxide inhibited the 2pY binding of Syk-tSH2 in a time-dependent as well as in a concentration-dependent manner similar as that seen for ZAP-tSH2. These data also indicate that Syk-tSH2 inhibition by these compounds is due to covalent adduct formation.

There are three cysteine residues that are conserved between the tandem-SH2 module of ZAP-70 and Syk (Cys 39 in ZAP-70 is not conserved in Syk, as noted earlier). We replaced two of these conserved cysteine residues in Syk by serine individually, and found that these mutant proteins were still sensitive to inhibition by compounds **B** and **C** (Supplement 6). As noted above, replacement of Cys 254 in ZAP-70 by serine (corresponding to Cys 299 in Syk) does not alter the extent of inhibition of ZAP-70 by the compounds (Supplement 2). This focused attention on Cys 206, which is unique to Syk and is located in the phosphotyrosine-binding pocket of the C-SH2 domain (Figure 7).

We replaced Cys 206 with serine or alanine in Syk-tSH2 and tested the sensitivity of this mutant to inhibition by compound **B** and **C**. Mutation of Cys 206 to serine or alanine protected Syk-tSH2 from inhibition by the compounds over 30 minutes, much as the C39S mutation protected ZAP-tSH2 (Figure 6). Additionally, C206S protected the protein from hydrogen peroxide inhibition at all concentrations. These experiments clearly demonstrate that Cys 206 in Syk is responsible for the inhibition of ITAM-binding by adduct formation or oxidation.

To further confirm that Cys 206 is indeed modified by compound, we performed mass spectrometry on Syk-tSH2. There are four cysteine residues in the tandem-SH2 module of Syk. The mass spectrometric analysis shows that Compound **B** forms four adducts on wildtype Syk-tSH2 but only three adducts on Syk-tSH2 C206S. Thus, Cys 206 is a site of covalent modification by compounds **B** (Supplement 7 and summarized in Supplement 8).

Introduction of a cysteine residue into the N-terminal phosphotyrosine-binding site of Syk confers sensitivity to inhibition

We have thus far shown that Cys 39 in ZAP-tSH2 and Cys 206 in Syk-tSH2 are critical for inhibition of ITAM binding by covalent cysteine modification. To further verify this mechanism of inhibition, we engineered a Syk-tSH2 construct that had a cysteine residue configuration that mimicked that of ZAP-tSH2. We began with the Syk construct in which Cys 206 was replaced by alanine, thus removing the one thiol group in the phosphotyrosine binding site of the C-SH2 domain. This Syk construct is insensitive to compound inhibition, as shown above. We then replaced Ser 44 in Syk, which corresponds to Cys 39 in ZAP-70, by cysteine. One would predict that if the presence of a cysteine residue in that location was both necessary and sufficient, then this ZAP-70-like engineered Syk construct should recover compound and hydrogen peroxide inhibition.

Syk-tSH2 C206A/S44C recovered its sensitivity to inhibition by both compounds **B** and **C** (Figure 8). When treated with hydrogen peroxide, Syk-tSH2 C206A/S44C also exhibited time-dependent inhibition. A reciprocal experiment in which a cysteine residue is introduced into the C-SH2 domain of ZAP-70 was attempted, but we were unable to express this construct.

Specificity of ZAP-70 inhibition by covalent modifiers

We also tested the ability of two common thiol-reactive alkylating agents, iodoacetamide (IAM) and n-ethylmaleimide (NEM), to inhibit ITAM binding. At all concentrations up to 1 mM, neither IAM nor NEM were able to inhibit ZAP-tSH2 (Figure 9). We then alkylated ZAP-tSH2 through an overnight incubation with a 10-fold molar excess of either IAM or NEM. After quenching with DTT and subsequent washes to remove excess compound and DTT, we tested binding of IAM or NEM-treated ZAP-tSH2 in the fluorescence polarization 2pY binding assay. In agreement with the time-dependence experiments, incubation with IAM or NEM did not affect the binding affinity between ZAP-tSH2 and 2pY. The measured K_D values of 10 nM for IAM-modified ZAP-tSH2 and 11 nM for NEM-modified ZAP-tSH2 is nearly identical to published values (Supplement 9) [34].

Discussion

In light of the fact that T-cell signaling is subject to redox regulation, it is not entirely surprising that ZAP-70, a crucial signaling molecule in the adaptive immune system, could be acting as a redox sensor. A previous study had shown that cultured primary T-lymphocytes exhibit decreased ZAP-70 phosphorylation upon treatment with peroxide and subsequent activation with α CD3 antibody [35]. We have shown here that the tandem-SH2 domains of ZAP-70 and Syk are sensitive to oxidation by hydrogen peroxide, providing a

potential mechanism by which ZAP-70 and Syk could be released from phosphorylated ITAM motifs through a negative feedback loop.

We analyzed the sequences of Syk and ZAP-70 proteins across the span of metazoan evolution, and built homology models to look at the positions of cysteine residues within the 3D structures. While the inter-SH2 domain linker of these Syk homologues do not show a high level of sequence identity, sequence alignments and homology models demonstrate that certain amino acid positions that form the hydrophobic helix-packing interactions of this linker are well-conserved. The presence of a cysteine residue at the position corresponding to Cys 206 in Syk appears to have arisen early in evolution. For example, a ZAP-70/Syk-like kinase can be found in porifera (*A. queenslandica*). This protein contains a considerably well-conserved tandem-SH2 unit (57% similar to human for the entire tandem-SH2 module). The C-SH2 domain of the porifera protein contains a cysteine residue (Cys 201) positioned within the sequence as Cys 206 is in human Syk. This cysteine residue is then lost in cnidaria (*H. vulgaris*), which does not appear to have cysteine residues in either phosphotyrosine-binding pockets. Mollusc (*A. californica*) Syk contains a cysteine residue (Cys 236) in an alternative position in the C-SH2 phosphotyrosine binding pocket, which might inhibit ITAM peptide binding upon oxidation. Continuing across the phylogenetic tree, echinoderm (*S. purpuratus*) does not contain any cysteine residues in either phosphotyrosine binding pocket.

Other protein-protein interaction domains are subject to oxidation and intramolecular disulfide bond formation that affects their affinity for ligand. PDZ domains are protein-protein interaction domains that have been shown to be redox regulated. Two cysteine residues in the peptide binding domain form an intramolecular disulfide bond that greatly enhances affinity for peptide ligand [36]. The T-cell signaling molecule ADAP contains two helically extended SH3 domains which bind lipids rather than polyproline motifs. NMR structural characterization and lipid binding assays showed that the N-terminal SH3 domain of ADAP forms an intramolecular disulfide bond, resulting in a conformational change and decreased affinity for lipids [37].

Intermolecular disulfide-induced dimerization is yet another means of inhibiting protein-protein interaction domains. The Src kinase domain as well as three FGFR family members contain a cysteine residue in their glycine-rich loops. Oxidation of these cysteine residues results in a dimerization of the kinase domains and reduced catalytic activity [38]. Disulfide-induced dimerization of SH2 domains has also been shown to inhibit their binding activity [39].

SH2 domain inhibitors are typically weakly binding peptide-mimetics or contain negatively charged phosphotyrosine mimetics, rendering SH2 domains intractable targets of inhibition by small molecules [40, 41]. A possible way of bypassing the weak affinity of SH2 domain inhibitors is by covalent inhibition. A previous study developed reversible covalent peptidic inhibitors of the SHP-1 SH2 domain [42]. That the covalent compounds that we have identified inhibit the tandem-SH2 modules of ZAP-70 and Syk provides a possible strategy for identifying clinically relevant inhibitors of ZAP-70 and Syk. The inability of n-ethylmaleimide or iodoacetamide to inhibit the binding of ZAP-tSH2 to 2pY peptide

suggests that there exists a minimal size or shape requirement necessary for inhibition by covalent adduct formation.

Inhibition of the tandem-SH2 domains of ZAP-70 and Syk binding to 2pY by hydrogen peroxide begs the question of whether the kinase domain of ZAP-70 is affected by hydrogen peroxide oxidation. Initial experiments from our lab show that hydrogen peroxide does not affect the kinase activity of the isolated kinase domain of ZAP-70 or the full-length protein toward a LAT-derived peptide substrate (Figure 10). This work is currently ongoing.

By identifying covalent inhibitors of the SH2 domains of ZAP-70 and Syk, we have identified cysteine residues that are sensitive to modification. This mechanism led us to postulate that inhibition through covalent cysteine modification could be recapitulating a cellular mechanism of inhibiting ZAP-70 and Syk through cysteine residue oxidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

H₂O₂	hydrogen peroxide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
IAM	iodoacetamide
ITAM	Immunoreceptor Tyrosine-based Activation Motif
LAT	Linker for activation of T-cells
Lck	Leukocyte-specific kinase

NEM	n-ethylmaleimide
SH2	Src-homology domain 2
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
Syk	Spleen tyrosine kinase
TCEP	tris(2-carboxyethyl)phosphine
ZAP-70	Zeta-chain associated protein of 70 kDa

Summary

Hydrogen peroxide and thiol-reactive inhibitors can attenuate the binding of the tandem-SH2 domains of ZAP-70 and Syk to doubly phosphorylated ITAM. This suggests a novel mechanism whereby oxidation of cysteine residues in these kinases could regulate immune cell signaling.

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Figure 1.

Structure and domain architecture of ZAP-70 and Syk. (**A, C**) ZAP-70 (PDB ID: 4K2R) and Syk (PDB ID: 4FL2) consist of two SH2 domains connected by a coiled-coil linker (inter-SH2 linker or interdomain A). This SH2 assembly is connected to the kinase domain by a mostly unstructured linker (SH2-kinase linker or interdomain B). (**B, D**) Ribbon diagram of ZAP-70 and Syk. Cysteine residues are numbered and α carbons of each cysteine residue are shown as a red spheres.

Figure 2.

(A) Chemical structures of compounds A, B, and C and adducts A, B, and C. Cysteine residues are shown in grey. (B-D) Deconvoluted mass spectra of different compounds incubated with ZAP-tSH2. Peaks of 31147 Da and 30569 Da correspond to unlabeled tandem-SH2 module (residues 1-259) and a cleavage product with a C-terminal segment missing (residues 1-253), respectively. Peaks with larger masses correspond to ZAP-tSH2 with mass additions due to covalent modification.

Figure 3. Structure of ZAP-tSH2:compound A. (A) Overall structure of ZAP-tSH2:compound A shows a novel conformation of the interdomain A inter-SH2 linker. Inset: 2Fo-Fc map shows electron density for Cys 117-adduct A. Deformation of the inter-SH2 domain linker helix backbone hydrogen bond is compensated for by hydrogen bond formation between the Asp 120 carbonyl and the secondary amine of the guanidino group on Arg 124. (B) Pi-stacking interaction between Cys 117-adduct C and Tyr 176 in an adjacent molecule contributes toward inter-SH2 domain linker coil deformation. (C) Superposition of ZAP:tSH2:compound A structure with that of ZAP-tSH2:ITAM (PDB ID: 2OQ1) shows bound sulfate ions in same position as ITAM phosphotyrosine residues.

Figure 4. Structure of ZAP-tSH2:ITAM:compound **B**. (A) Structure of ZAP-tSH2:ITAM:compound **B** overlaid with that of ZAP-tSH2:ITAM previously determined (PDB ID: 2OQ1, shown in grey). (B) Structure of ZAP-tSH2:ITAM:compound **B**. Inset: 2Fo-Fc map shows electron density for adduct **B** formed on Cys 78.

Figure 5.

(A) Compounds A, B and C inhibit ITAM binding in a time-dependent manner. (B) C39S mutation protects ZAP-tSH2 from compound B and C but not A inhibition. (C) ZAP-tSH2 is subject to inhibition by non-reducing conditions. Titrating down reductant increases the rate of inhibition. (E) Addition of hydrogen peroxide inhibits 2pY peptide binding. (D, F) C39S mutation protects from inhibition due to non-reducing conditions as well as by hydrogen peroxide.

Figure 6. (A-B) Compounds and hydrogen peroxide inhibit Syk-tSH2 binding to 2pY peptide. (C-D) C206S mutation protects from compound and hydrogen peroxide inhibition. (E) C206A mutant demonstrates a similar lack of inhibition to compounds.

Figure 7.
(A-B) Rendering is of an overlay of ZAP-70 N-SH2 (above, blue) and Syk C-SH2 (below, purple) domains shown separately. The specific cysteine residue targets of covalent inhibition for both proteins are in a position that would block phosphotyrosine binding.

Figure 8.

(A) Engineered Syk with ZAP-70-like cysteine residue configuration regains inhibition to compounds **B** and **C**. (B) Engineered Syk-tSH2 is also sensitive to inhibition by H₂O₂.

Figure 9.
(A-B) Neither n-ethylmaleimide (NEM) nor iodoacetamide (IAM) inhibit ZAP-tHS2:2pY binding.

Figure 10.

The addition of H₂O₂ to a FRET-based assay of kinase activity does not affect the rate of ZAP-70 kinase activity. Activity is expressed as a ratio of acceptor fluorescence over donor fluorescence.