Published in final edited form as:

J Proteome Res. 2015 May 1; 14(5): 2082–2089. doi:10.1021/pr501172u.

Wide-scale quantitative phosphoproteomic analysis reveals that cold treatment of T cells closely mimics soluble antibody stimulation

Qinqin Ji[#] and Arthur R. Salomon^{*,#,\$}

[#] Department of Chemistry, Brown University, Providence, RI 02912, United States

^{\$} Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02903, United States

Abstract

The activation of T-lymphocytes through antigen-mediated T-cell receptor (TCR) clustering is vital in regulating the adaptive-immune response. Although T cell receptor signaling has been extensively studied, the fundamental mechanisms for signal initiation are not fully understood. Reduced temperature initiated some of the hallmarks of TCR signaling such as increased phosphorylation and activation on ERK and calcium release from the endoplasmic reticulum as well as coalesce T-cell membrane microdomains. The precise mechanism of TCR signaling initiation due to temperature change remains obscure. One critical question is whether signaling initiated by cold treatment of T cells differs from signaling initiated by crosslinking of the T cell receptor. To address this uncertainty, a wide-scale, quantitative mass spectrometry-based phosphoproteomic analysis was performed on T cells stimulated either by temperature shift or through crosslinking of the TCR. Careful statistical comparison between the two stimulations revealed a striking level of identity between the subset of 339 sites that changed significantly with both stimulations. This study demonstrates for the first time, at unprecedented detail, that T cell cold treatment was sufficient to initiate signaling patterns nearly identical to soluble antibody stimulation, shedding new light on the mechanism of activation of these critically important immune cells.

Keywords

Cold stimulation; Jurkat; T cell signaling; Immunology; Mass spectrometry; Phosphoproteome

INTRODUCTION

Signals from recognition of peptide-MHC complexes by T-cell receptor (TCR) are important for T-cell development, survival, and death, as well as subset lineage specification and differentiation into effector or memory T cells in response to foreign antigens.¹⁻² TCR

*Corresponding Author Tel: (401)863-6091. Fax: (401)863-6087. art@drsalomon.com (A. Salomon).

Notes

The authors declare no completing financial interest.

signaling is initiated through engagement of the TCR by a cognate peptide-MHC molecule, resulting in sequential activation of Src kinase Lck and Fyn, which phosphorylates the ζ -chain immunoreceptor tyrosine-based activation motifs (ITAMs).³ Phosphorylated ITAMs recruit and activate the Syk family protein kinase ZAP-70, which then phosphorylates the adaptor protein LAT and SLP-76, forming a signalosome complex essential for the assembly of downstream signaling proteins.⁴⁻⁵

Although the molecular events and the protein components that are involved in TCR signaling have been extensively studied, fundamental questions like the mechanisms for signaling initiation and early signaling transduction are still controversial. *In vivo*, T cell signaling is initiated through the binding of the T cell receptor with MHC-peptide presented on the surface of antigen presenting cells leading to productive clustering of T cell signaling proteins.⁶ The strength of stimulation of the T cell receptor is an important context to understand the physiologically relevant responses of these cells and the use of antibodies leads to levels of stimulation stronger than observed *in vivo*.⁷ Soluble antibodies, such as anti-CD3/CD4 antibody engage with receptor and are used for receptor crosslinking to initiate TCR signaling pathways leading to activation of many of the same downstream effectors as the physiological stimulation with MHC-peptide.⁷⁻⁸ Aggregation of lipid rafts through aggregation of the ganglioside GM1 using cholera toxin b subunit and anti cholera toxin also leads to many of the hallmarks of T cell activation.⁹ Interestingly, without ligation of receptor by soluble antibody, low temperature alone is able to induce coalescence of membrane microdomains and activation of signaling pathways, although the precise mechanism of activation was not fully resolved in these studies.¹⁰⁻¹¹ An important downstream event in TCR signaling pathway, tyrosine phosphorylation of ERK was observed both in Jurkat cells and human primary T cells after reduced temperature treatment.¹⁰⁻¹¹ Immune blotting with the pan-phosphotyrosine specific antibody 4G10 revealed that other T cell signaling proteins including LAT, Lck, Fyn Src-family PTKs, ZAP70 may show increased tyrosine phosphorylation in Jurkat cells when treated with low temperature alone although information about individual sites of phosphorylation was not revealed in this data.¹⁰ The increased phosphorylation of T cell signaling proteins was reversible when cells were switched from low temperature back to physiological temperature (37 °C). Furthermore, low temperature-induced stimulation proved sufficient to evoke an increase in intracellular free Ca^{2+} concentrations, one of the obligatory events during T-cell activation.¹¹

As T cell signaling is controlled by the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs),¹² cold-induced activation could at least hypothetically be due to a differential impact of temperature shift on the kinetics of individual PTKs and PTPs, leaving the former relatively more active and latter relatively less active at low temperature. The impact of temperature on the specific kinetics of individual kinases and phosphatases in T cells has not been specifically studied. Other researchers have proposed that cold-induced coalescence of T-cell plasma membrane microdomains activates signaling pathways. In these studies, at 37 °C, Lck and CD3 were evenly distributed while cold treatment induced a patchy plasma membrane distribution where CD3 and Lck were

considerably colocalized.¹¹⁻¹² These studies support the important role of membrane microdomains in TCR signaling.

Although some progress has been made in investigating a limited number of signaling events induced by low temperature treatment of T cells, a comprehensive comparison between signaling initiated with temperature shift or through crosslinking of the TCR could shed new light onto the precise mechanisms of signaling initiation. A central question is whether the signaling events initiated by specific crosslinking of the T cell receptor with antibody are in any way divergent from the pathways initiated by cold stimulation induced rearrangement of lipid microdomains. Here we report a wide scale quantitative analysis of 1344 unique sites of tyrosine phosphorylation observed in T cells stimulated by cold shift or through receptor crosslink. This analysis revealed that the phosphorylation signaling networks induced by receptor crosslinking using soluble anti-CD3/CD4 antibody, are nearly identical to cold-induced coalescence of T cell plasma membrane microdomains.

EXPERIMENTAL SECTION

Cell Culture, Treatment and Lysis

Jurkat E6-1 cells were obtained from American Tissue Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% heat-inactivated undialyzed FBS (Hyclone, Logan, UT), 2mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator with 5% CO₂ at 37 °C. Cells were then washed and re-suspend in PBS for further treatment. Cells were incubated for 20 minutes at 4 °C for cold stimulation. As a control, cells were incubated at 37 °C for 20 minutes. For soluble antibody stimulation, cells were treated with anti-CD3 and anti-CD4 antibody in PBS (clones OKT3 and OKT4; eBioscience, San Diego, CA) for 5 minutes following incubation at 37°C for 20 minutes as described.¹³ Once treatment was completed, cells were lysed in lysis buffer (9 M urea, 1 mM sodium orthovanadate, and 20 mM HEPES, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, pH 8.0).

Protein reduction, Alkylation, Digestion and Peptide Immunoprecipitation

Protein concentration was measured by the DC Protein Assay (Bio-Rad, Hercules, CA). Reduction with DTT, alkylation with iodoacetamide, and digestion with trypsin were performed as previously described.¹³ Tryptic peptides were desalted using C18 Sep-Pak plus cartridges (Waters, Milford, MA) and lyophilized for 48 hours to dryness.¹⁴ Peptide immunoprecipitation was performed using pre-conjugated p-Tyr-100 phosphotyrosine antibody beads (Cell Signaling Technology) as previously described.¹³ Particularly, a 5 pmol fraction of synthetic phosphopeptide LIEDAEpYTAK was added to each sample as an exogenous quantitation standard prior to peptide immunoprecipitation. After immunoprecipitation, samples were then desalted using C18 Zip Tip pipette tips (Millipore Corporation Billerica, MA) according to manufacturer's instructions.

Automated nano-LC/MS and Data Analysis

Tryptic peptides were analyzed by a fully automated phosphoproteomic technology platform.¹⁵⁻¹⁶ Phosphopeptides were eluted into a Linear Trap Quadrupole (LTQ) Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) through a PicoFrit analytical column (360 μ m outer diameter 75 μ m inner diameter-fused silica packed on a pressure bomb with 15 cm of 3- μ m Monitor C18 particles; New Objective, Woburn, MA) with a reversed phase gradient (0-70% 0.1M acetic acid in acetonitrile in 90 minutes). An electrospray voltage of 2.0 kV was applied using a split flow configuration, as described previously.¹⁷ Spectra were collected in positive ion mode and in cycles of one full MS scan in the Orbitrap (m/z : 400-1800), followed by data-dependent MS/MS scans in the LTQ, sequentially of the ten most abundant ions in each MS scan with charge state screening for +1, +2, +3 ions and dynamic exclusion time of 30 seconds. The automatic gain control was 1,000,000 for the Orbitrap scan and 10,000 for the LTQ scans. The maximum ion time was 100 milliseconds for the LTQ scan and 500 milliseconds for the Orbitrap full scan. Orbitrap resolution was set at 60,000.

MS/MS spectra were searched against the non-redundant UniProt complete proteome database (UNIPROT database released 2013/02/01) containing 87,613 forward and an equal number of reversed decoy protein entries using the Mascot algorithm version 2.2.07 from Matrix Science.¹⁸ Peak lists were generated using extract msn.exe (07/12/07) using a mass range of 600-4500. The Mascot database search was performed with the following parameters: trypsin enzyme specificity, 2 possible missed cleavages, 7 ppm mass tolerance for precursor ions, 0.5 Da mass tolerances for fragment ions. Search parameters specified a differential modification of phosphorylation (+79.9663 Da) on serine, threonine, and tyrosine residues, a dynamic modification of methionine oxidation (+15.9949 Da), and a static modification of carbamidomethylation (+57.0215 Da) on cysteine. Mascot results were filtered by Mowse Score (>20) and precursor mass error (<2ppm). FDR was estimated with the decoy database approach after final assembly of non-redundant data into heatmap by a logistic spectral score as described.¹⁹⁻²⁰ The Ascore algorithm²¹ was applied to all data, and all reported phosphorylation site positions reflected the top Ascore prediction.

Relative Phosphopeptide Abundance Quantification

Relative quantification of phosphopeptide abundance was performed via calculation of select ion chromatogram (SIC) peak areas for phosphopeptides. Retention time alignment of individual replicate analyses was performed as described previously.²² Peak areas were calculated by inspection of SICs using software programmed in Microsoft Visual Basic 6.0 based on Xcalibur Development kit 2.1 (Thermo Fisher Scientific). This approach employed the ICIS algorithm available in the Xcalibur XDK with the following parameters: multiple resolution of 8, noise tolerance of 0.1, noise window of 40, scans in baseline of 5, and inclusion of refexc peaks parameter value, which is false. SIC peak areas were determined for every phosphopeptide that was identified by MSMS (see Figure S1 for a representative phosphopeptide analysis). In the case of a missing MSMS for a particular peptide, in a particular replicate or cell treatment, SIC peak areas were calculated according to the peptides' isolated mass and the retention time calculated from retention time alignment.²³ SIC peak areas of individual peptides were normalized to the peak area of exogenously

spiked phosphopeptide LIEDEpYTAK added in the same amount to each replicate experiment and accompanied cellular phosphopeptides through phosphopeptide enrichment and reversed-phase elution into the mass spectrometer. A minimum SIC peak area equivalent to the typical spectral noise level of 1000 was required of all data reported for quantification.

Temporal label free heatmaps were generated as previously described.¹⁴ Ratio heatmaps were then generated from 5 biological replicate experiments with color reflecting fold changes of average peak area of phosphopeptides between stimulated cells (cold stimulation or anti-CD3/4 antibody stimulation) and control. For a given phosphopeptide, a black color represents a fold change of 1 between stimulated cells and control. A red color represented less abundance of the given phosphopeptide in stimulated cells than control, and green color indicates the opposite. The magnitude of change of the heatmap color was calculated as described.¹⁵ To select phosphopeptides that show statistically significant differential abundance between stimulated cells and control, two-tailed unpaired Student's *t* tests and *q* values for multi-test control were calculated from replicated data (*n* = 3) for each phosphopeptide as previously described.²⁴⁻²⁵ Cutoffs for phosphopeptides deemed as significantly changed were *q* values < 0.05 as well as a fold change >2-fold or <0.5-fold for both CD3/4 versus control and cold stimulation versus control. In the case of multiple peptide isoforms containing a given phosphorylation site that meet the significance thresholds (different peptide cleavage states, charge states, or methionine oxidation states), the peptide isoform with the highest average peak area in the control treatment was selected. Table S2 contains a comprehensive list of all phosphopeptide isoforms detected at a 1% FDR and the associated quantitation. The remaining figures and discussion within the paper only reference the selected peptide isoform according to the selection criteria. Statistically significant changes are denoted by white dots on the heatmap square.

Western blotting

Total protein extracts were prepared as described previously.¹³ Immunoblots were performed on equal amounts of protein extracts as previously described using Odyssey CLx Imaging System (Li-Cor).¹³ With the exception membranes were probed with the following primary antibodies: anti-phospho-p44/p42 MAPK (ERK1/2)(Thr202/Tyr203), anti-p44/p22 MAPK (ERK1/2) (above antibodies from Cell Signaling Technologies, Danvers, MA), and clone 4G10 (EMD Millipore).

Results and Discussion

Quantitative phosphoproteome analysis

To systematically identify the phosphorylation events induced by reduced temperature, an MS-based quantitative phosphoproteomic strategy was applied to explore the complex phosphorylation signaling networks. A total of 5 biological replicate experiments were analyzed: cells incubated in 37 °C for 20 minutes (Control, *n*=5), cells incubated at 4 °C for 20 minutes (Cold, *n*=5), cells incubated in 37 °C for 20 minutes followed by anti-CD3 and anti-CD4 antibody stimulation for 5 minutes (CD3/4 stimulation, *n*=5). The experimental data analysis flow-chart is shown in Figure 1A-B. A complete list of tyrosine

phosphorylated peptides with Mowse score > 20 and mass error <2 ppm including reversed database hits from every LC-MS sample was provided (Table S1). Through analysis of 5 biological replicates, 1344 unique tyrosine phosphorylation sites assigned to 862 unique proteins were identified at 1% FDR (estimated by decoy database approach) and quantified (Table S2). The distribution of ratios of uniquely assigned phosphopeptides indicated that cold stimulation resulted in a wider range of fold changes, while CD3/4 stimulation resulted in a more sharp distribution (Figure S2A). The detection of significantly altered phosphopeptides was carried out applying student's *t* test to the biological replicate peak areas corrected for multiple hypotheses by *q* value. Fold change of peak area for each phosphopeptide was calculated between cold stimulated cells or CD3/4 stimulated cells towards control. The distribution of fold changes across all phosphopeptides allowed for the calculation of an inflection point and selection of a minimal fold change (Figure S2B). Therefore, to be called significant, a phosphopeptide was required to pass two criteria: *q* value < 0.05 and fold change > 2-fold or < 0.5-fold for both ratios (CD3/4 versus control and cold stimulation versus control). Across all data collected, there was only one example of significantly changed phosphorylation sites with different peptide isoforms indicating inverted fold changes (Aldolase Tyr5). This site was omitted from all discussion. Figure 1C depicts volcano plots for the *q* value, corrected for multiple testing, versus log-ratios. A substantial number of significantly changed phosphopeptides (*q* value < 0.05) and with fold change >2-fold or <0.5-fold were detected. This analysis also revealed that most tyrosine sites showed increased phosphorylation upon cold stimulation or CD3/4 stimulation (Figure 1C).

There are 660 unique phosphotyrosine sites assigned to 446 unique proteins showing a statistically significant alteration between cold stimulated cells and control cells (*q* value < 0.05; >2-fold or <0.5-fold) and 313 unique phosphotyrosine sites on 448 proteins showing significant alteration between CD3/4 antibody stimulation and control cells. In total, 337 unique phosphorylation sites assigned to 235 unique proteins showed a statistical significant change (*q* value < 0.05; > 2-fold or < 0.5-fold) in both cold stimulation and CD3/4 stimulation. Of these 337 unique tyrosine phosphorylation sites, 97.6% (329 sites) showed identical direction of significant change with either cold or CD3/4 stimulation (Table S3). The 8 sites that showed inverted significant changes were generally found on proteins of unknown function in T cells (Table S3).

The subset of proteins annotated as KEGG TCR signaling and actin cytoskeleton showed nearly identical changes when comparing cold stimulation to CD3/4 stimulation. Ratio heatmaps were generated for phosphotyrosine sites identified with statistically significant changes upon two stimulations for visualization of global effects on the canonical TCR signaling (Figure 2 and Figure S3). Within the canonical T cell signaling pathway, phosphorylation sites significantly elevated by CD3/4 stimulation were nearly identical to cold stimulation with the only exception being a single biologically uncharacterized site at Try69 within the SH2 domain of ZAP-70 that showed decreased phosphorylation upon CD3/4 stimulation and increased phosphorylation upon cold stimulation (Figure 2). In addition, all of the KEGG actin cytoskeleton signaling pathway proteins displayed the same direction of significant change with either stimulation (Table 1).

Cold stimulation mimicked elevated phosphorylation alteration from CD3/4 stimulation on canonical TCR signaling modules

Increased phosphorylation was observed on proximal signaling molecules such as CD3, TCR ζ chain, and the kinase Lck and ZAP-70 compared to control (Figure 2). An initial downstream signaling event downstream of the TCR is the activation of the tyrosine kinase Lck by modification of phosphorylation at specific residues.²⁶⁻²⁷ The activation site of Lck, Tyr394, was found to be increased upon stimulation, with a fold change greater than 30-fold by cold stimulation and greater than 8-fold change by CD3/4 stimulation compared to control. Phosphorylation of Tyr192, which leads to inhibition of Lck association with its substrates through its SH2 domain,²⁷ had greater than 10-fold change by cold stimulation and greater than 3-fold change by CD3/4 stimulation. Active Lck phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in the ζ chain of the TCR complex, leading to recruitment of the tyrosine-protein kinase ZAP-70 and its subsequent phosphorylation by Lck (Figure 2).²⁸ Five sites on ζ , known to be substrates of Lck, were similarly elevated with either stimulation. Tyrosine sites on ZAP-70 also showed statistically elevated phosphorylation in both cold stimulation and CD3/4 stimulation. One of these sites included Tyr292, known to be regulated by autophosphorylation, was found to display greater than 24-fold elevated phosphorylation with cold stimulation and CD3/4 stimulation. Tyr69 on ZAP-70 with unknown biological function, showed a greater than 10-fold increase with cold stimulation, but had a decreased phosphorylation with CD3/4 stimulation.

Upon activation, ZAP-70 phosphorylates SLP-76 and LAT, serving as scaffolds for formation of a common signalosome complex. Only phosphorylation of Tyr532 on SLP-76, with unknown biological function was quantified and found to be reduced with either stimulation. Downstream of the TCR, proteins associated with SLP-76 and LAT also showed elevated phosphorylation in stimulated cells. The Tec kinase Itk had over 50-fold increased phosphorylation on its active site Tyr512 with cold stimulation compared to over 11-fold increase with CD3/4 stimulation. Tyr771 on PLC γ 1, known to be phosphorylated after TCR engagement, showed elevated phosphorylation with cold stimulation and CD3/4 stimulation.

Downstream signaling events in the TCR pathway including activating phosphorylation of MAPK, such as on p38 α MAPK, and ERK1/2 were observed. Tyr182 on p38 α had greater than 3-fold change with cold stimulation and CD3/4 stimulation. ERK1/2 phosphorylation showed greater than 15-fold change with cold stimulation and CD3/4 stimulation.

In order to validate the quantitative phosphoproteomic analysis, immunoblots were performed to assay the phosphorylation of selected members of the TCR signaling pathway. Phosphorylation of ERK1/2 was observed to be elevated with cold stimulation and CD3/4 stimulation (Figure S4A). Consistent with previous reports,¹⁰ phosphorylation on a large range of proteins was increased in response to cold stimulation and CD3/4 stimulation when visualized with 4G10 immunoblot (Figure S4B).

Cold stimulation and CD3/4 stimulation both lead to increased phosphorylation on canonical TCR actin cytoskeletal associated proteins

The actin cytoskeleton plays an important role in dynamic processes such as cell motility, cytokinesis, and phagocytosis. Multiple signaling proteins control the rearrangement of the actin cytoskeleton necessary for formation of the immunological synapse.²⁹ The results of this quantitative phosphoproteomic analysis also revealed that cold stimulation exactly mimicked the pattern of elevated phosphorylation induced by CD3/4 stimulation on KEGG actin cytoskeletal pathway proteins (Table 1). A total of 18 unique tyrosine phosphorylation sites assigned to 15 unique proteins were significantly elevated with both stimulations (Table 1). Examples of regulators of actin cytoskeletal dynamics that showed statistically significant increases in phosphorylation in cold stimulation and CD3/4 stimulation include CRKL (Tyr251), IGF-1 receptor (Tyr108), Paxillin (Tyr88, Tyr188), Profilin 1 (Tyr129) and ROCK2 (Tyr722). Tyrosine phosphorylation of Vav leads to activation of its guanine nucleotide exchange factor (GEF) activity and leads to the activation of Rac and Cdc42.³⁰ Phosphorylation of Tyr791 on Vav was increased with cold stimulation and CD3/4 stimulation. Furthermore elevated phosphorylation on Tyr256 of WASP was observed with cold stimulation and CD3/4 stimulation. WASP is a key cytoskeletal regulator in hematopoietic cells. Fyn-regulated phosphorylation of Tyr291 at WASP after TCR ligation has been demonstrated to be necessary for WASP effector activities downstream of the T cell receptor.³¹⁻³²

CONCLUSIONS

In this study, signaling induced by cold treatment of T cells was investigated and also compared to signaling induced by soluble antibody stimulation using quantitative phosphoproteomics. Both stimulations lead to nearly identical increased tyrosine phosphorylation on a wide range of T cell signaling proteins including the KEGG TCR signaling and actin cytoskeletal groups of proteins. The high degrees of overlap in the patterns of tyrosine phosphorylation within the KEGG TCR and actin cytoskeletal groups of proteins suggest a high degree of mechanistic overlap in the initiation of T cell signaling with either cold treatment or CD3/4 stimulation. Previous studies indicated that Lck was required for the signaling initiated by antibody³³ as well as by cold treatment, in the Lck-deficient cell line JCaM1.6.¹⁰ In addition, our data does not support the hypothesis that cold-induced activation is caused by a general perturbation of the kinetics of PTKs and PTPs in a nonspecific manner. Although the magnitude of the fold changes in phosphopeptide abundance was wider for cold stimulation compared to CD3/4 stimulation, this could be explained by differences in the number of receptor molecules that become activated with the different stimulation protocols. The fact that cold stimulation affected 97.6% of all of the significantly changed sites in the same direction as CD3/4 stimulation supports the conclusion that cold stimulation activates the same pathways as direct receptor crosslink with antibody. Future studies should further investigate the similarities between cold stimulation and stimulation of T cells by peptide-MHC or via association with antigen presenting cells. For decades, ligation of TCR was believed to cause its translocation to lipid rafts, where TCR signaling is initiated. Finally, as incubation of cells in low temperature is a standard procedure in cell biology, this study highlights the possibility of unintended

activation of a cellular receptor under these conditions, which could complicate the interpretation of data. In this context, caution is also warranted with cold stimulation of other cell types dependent upon signaling pathways involving rearrangement of lipid rafts such as adaptive and innate immune cells, and transformed cell lines.³⁴⁻³⁵ This study also highlights the importance of future studies of T cell activation in the context of exposure of the skin to cold temperatures and of transplanted organs transported on ice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

The authors wish to acknowledge financial support from NIH grant R01 AI083636. In addition, this research is based in part upon work conducted using Rhode Island NSF/EPSCoR Proteomics Share Resource Facility, which is supported in part by the National Science Foundation EPSCoR Grant No. 1004057, National Institute of Health Grant No. 1S10RR020923, a Rhode Island Science and Technology Advisory Council grant and the Division of Biology and Medicine, Brown University.

ABBREVIATIONS

ACTA1	Actin alpha, cardiac muscle
ADAP	adhesion and degranulation adaptor protein
Cbl	casitas B-lineage lymphoma
CblB	casitas B-lineage lymphoma proto-oncogene b
CD28	cluster of differential 28
CRKL	Crk-like protein
DTT	dithiothreitol
Erk1/2	extracellular signal-regulated kinase-1/2
FDR	false discovery rate
Fyn	proto-oncogene tyrosine-protein kinase Fyn
Gab2	GRB2-associated-binding protein 2
IGF1	insulin-like growth factor 1 (IGF-1) receptor
ITAM	immunoreceptor tyrosine activation motif
ITGB1	integrin beta 1
Itk	IL2-inducible T-cell kinase
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LTQ	linear trap quadrupole
MAPK14	mitogen-activated protein kinase 14

NCK1	non-catalytic region of tyrosine kinase adaptor protein 1
PIKFYVE	Phosphoinositide kinase, FYVE finger containing
PLCγ1/2	phospholipase C gamma 1/2
PPP1R12A	protein phosphatase 1, regulatory subunit 12A
PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit
PTKs	protein tyrosine kinases
PTPs	protein tyrosine phosphatases
PYK2	protein tyrosine kinase 2 beta
ROCK2	Rho-associated protein kinase 2
SHP-1	SH2 domain-containing protein tyrosine phosphatase 1
TCR	T cell receptor
Tec	tyrosine protein kinase Tec
TIAM1	T-lymphoma invasion and metastasis-inducing protein 1
WASP	Wiskott-Aldrich syndrome protein
VAV1	proto-oncogene vav
ZAP-70	zeta-chain-associated protein kinase 70

REFERENCE

1. Krummel M, Wulfig C, Sumen C, Davis MM. Thirty-six views of T-cell recognition. Philosophical transactions of the Royal Society of London. Series B, Biological sciences. 2000; 355(1400):1071–6.
2. Acuto O, Di Bartolo V, Michel F. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. Nat Rev Immunol. 2008; 8(9):699–712. [PubMed: 18728635]
3. Zamoyska R, Basson A, Filby A, Legname G, Lovatt M, Seddon B. The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. Immunol Rev. 2003; 191(1): 107–118. [PubMed: 12614355]
4. Zhang W, Sloan-Lancaster J, Kitchen J, Tribble RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell. 1998; 92(1):83–92. [PubMed: 9489702]
5. Bubeck Wardenburg J, Fu C, Jackman JK, Flotow H, Wilkinson SE, Williams DH, Johnson R, Kong G, Chan AC, Findell PR. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J Biol Chem. 1996; 271(33):19641–4. [PubMed: 8702662]
6. Dustin ML, Groves JT. Receptor signaling clusters in the immune synapse. Annual review of biophysics. 2012; 41:543–56.
7. Corse E, Gottschalk RA, Allison JP. Strength of TCR-peptide/MHC interactions and in vivo T cell responses. J Immunol. 2011; 186(9):5039–45. [PubMed: 21505216]
8. Chakraborty AK, Weiss A. Insights into the initiation of TCR signaling. Nat Immunol. 2014; 15(9): 798–807. [PubMed: 25137454]
9. Janes PW, Ley SC, Magee AI. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J Cell Biol. 1999; 147(2):447–461. [PubMed: 10525547]

10. Magee AI, Adler J, Parmryd I. Cold-induced coalescence of T-cell plasma membrane microdomains activates signalling pathways. *J Cell Sci.* 2005; 118(14):3141–3151. [PubMed: 16014381]
11. Wurm S, Paar C, Sonnleitner A, Sonnleitner M, Hoglinger O, Romanin C, Wechselberger C. Co-localization of CD3 and prion protein in Jurkat lymphocytes after hypothermal stimulation. *Febs Lett.* 2004; 566(1-3):121–125. [PubMed: 15147880]
12. Mustelin T, Tasken K. Positive and negative regulation of T-cell activation through kinases and phosphatases. *The Biochemical journal.* 2003; 371:15–27. Pt 1. [PubMed: 12485116]
13. Helou YA, Nguyen V, Beik SP, Salomon AR. ERK Positive Feedback Regulates a Widespread Network of Tyrosine Phosphorylation Sites across Canonical T Cell Signaling and Actin Cytoskeletal Proteins in Jurkat T Cells. *Plos One.* 2013; 8(7):e69641. [PubMed: 23874979]
14. Nguyen V, Cao LL, Lin JT, Hung N, Ritz A, Yu KB, Jianu R, Ulin SP, Raphael BJ, Laidlaw DH, Brossay L, Salomon AR. A New Approach for Quantitative Phosphoproteomic Dissection of Signaling Pathways Applied to T Cell Receptor Activation. *Mol Cell Proteomics.* 2009; 8(11):2418–2431. [PubMed: 19605366]
15. Yu KB, Salomon AR. PeptideDepot: Flexible relational database for visual analysis of quantitative proteomic data and integration of existing protein information. *Proteomics.* 2009; 9(23):5350–5358. [PubMed: 19834895]
16. Yu KB, Salomon AR. HTAPP: High-throughput autonomous proteomic pipeline. *Proteomics.* 2010; 10(11):2113–2122. [PubMed: 20336676]
17. Ficarro SB, Salomon AR, Brill LM, Mason DE, Stettler-Gill M, Brock A, Peters EC. Automated immobilized metal affinity chromatography/nano-liquid chromatography/electrospray ionization mass spectrometry platform for profiling protein phosphorylation sites. *Rapid Commun Mass Sp.* 2005; 19(1):57–71.
18. Perkins DN, Pappin DJC, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 1999; 20(18):3551–3567. [PubMed: 10612281]
19. Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods.* 2007; 4(3):207–214. [PubMed: 17327847]
20. Yu KB, Sabelli A, DeKeukelaere L, Park R, Sindi S, Gatsonis CA, Salomon A. Integrated platform for manual and high-throughput statistical validation of tandem mass spectra. *Proteomics.* 2009; 9(11):3115–3125. [PubMed: 19526561]
21. Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat Biotechnol.* 2006; 24(10):1285–1292. [PubMed: 16964243]
22. Demirkan G, Yu K, Boylan JM, Salomon AR, Gruppiso PA. Phosphoproteomic profiling of in vivo signaling in liver by the mammalian target of rapamycin complex 1 (mTORC1). *Plos One.* 2011; 6(6):e21729. [PubMed: 21738781]
23. Ji Q, Ding Y, Salomon AR. SLP-76 N-terminal tyrosine residues regulate a dynamic signaling equilibrium involving feedback of proximal TCR signaling. *Mol Cell Proteomics.* 2014
24. Storey JD. The positive false discovery rate: A Bayesian interpretation and the q-value. *Ann Stat.* 2003; 31(6):2013–2035.
25. Storey JD. A direct approach to false discovery rates. *J Roy Stat Soc B.* 2002; 64:479–498.
26. Salmond RJ, Filby A, Qureshi I, Caserta S, Zamoyska R. T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunol Rev.* 2009; 228:9–22. [PubMed: 19290918]
27. Couture C, Songyang Z, Jascur T, Williams S, Tailor P, Cantley LC, Mustelin T. Regulation of the Lck SH2 domain by tyrosine phosphorylation. *J Biol Chem.* 1996; 271(40):24880–4. [PubMed: 8798764]
28. Huang YP, Wange RL. T cell receptor signaling: Beyond complex complexes. *J Biol Chem.* 2004; 279(28):28827–28830. [PubMed: 15084594]
29. Dustin ML, Cooper JA. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nat Immunol.* 2000; 1(1):23–9. [PubMed: 10881170]

30. Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature*. 1997; 385(6612):169–72. [PubMed: 8990121]
31. Blundell MP, Bouma G, Metelo J, Worth A, Calle Y, Cowell LA, Westerberg LS, Moulding DA, Mirando S, Kinnon C, Cory GO, Jones GE, Snapper SB, Burns SO, Thrasher AJ. Phosphorylation of WASp is a key regulator of activity and stability in vivo. *P Natl Acad Sci USA*. 2009; 106(37): 15738–15743.
32. Badour K, Zhang J, Shi F, Leng Y, Collins M, Siminovitch KA. Fyn and PTP-PEST-mediated regulation of Wiskott-Aldrich syndrome protein (WASp) tyrosine phosphorylation is required for coupling T cell antigen receptor engagement to WASp effector function and T cell activation. *J Exp Med*. 2004; 199(1):99–112. [PubMed: 14707117]
33. Davis SJ, van der Merwe PA. Lck and the nature of the T cell receptor trigger. *Trends Immunol*. 2011; 32(1):1–5. [PubMed: 21190897]
34. Kruger M, Kratchmarova I, Blagoev B, Tseng YH, Kahn CR, Mann M. Dissection of the insulin signaling pathway via quantitative phosphoproteomics. *Proc Natl Acad Sci U S A*. 2008; 105(7): 2451–6. [PubMed: 18268350]
35. Edidin M. The state of lipid rafts: from model membranes to cells. *Annual review of biophysics and biomolecular structure*. 2003; 32:257–83.

Figure 1.

Flow-chart of phosphoproteomics data acquisition and data analysis. A) Experiment design of this proteomics study. Two stimulations were performed on human Jurkat T cells, cold stimulation and soluble antibody (CD3/4) stimulation. B) Data analysis workflow. C) Volcano plots for q values versus intensity changes for cold stimulation and CD3/4 stimulation. Cyan points are considered to be significant, having q-values below 0.05 and fold change > 2 or < 0.5 .

Figure 2.

Ratio heatmaps of identified canonical TCR signaling proteins that showed significant changes of phosphopeptide abundance for both types of stimulation (q value < 0.05 ; > 2 -fold or < 0.5 -fold). Depicted is the canonical TCR signaling pathway with quantitative ratio heatmaps beside each protein, corresponding to the changes in phosphorylation between stimulated cells (cold or CD3/4 antibody stimulation) and control cells. Cold vs control is the heatmap square on the left of each pair while CD3/4 vs control is on the right. Data represent five biological replicate experiments. Green represents elevated phosphorylation in stimulated cells relative to control whereas red was elevated in control relative to stimulation. White dots within the heatmap indicate statistically significant differences.

Table 1

List of actin cytoskeletal proteins that showed significant changes of phosphopeptide abundance for both types of stimulation (q value < 0.05 ; > 2 -fold or < 0.5 -fold). Actin cytoskeletal proteins were determined through the KEGG Regulation of actin cytoskeleton pathway.

Protein Name	p-Sites	Cold vs. Control		CD3/4 vs. Control	
		Ratio ¹	Q value ²	Ratio	Q value
ACTA1	Y55*	3.9	4.16E-02	7.7	2.33E-02
CRKL	Y251	91.7	3.69E-05	26.8	2.96E-05
ERK1	T202Y204*	11.9	2.46E-02	7.0	3.27E-02
ERK1	Y204*	29.8	2.23E-05	78.3	2.16E-06
ERK2	T185Y187*	45.8	4.60E-04	76.2	1.74E-05
ERK2	Y187*	15.8	7.21 E-06	45.9	6.18E-06
IGF1	Y1165*	23.4	3.57E-04	7.5	3.93E-03
ITGB1	Y783	14.5	1.46E-06	2.1	2.41 E-03
PPP1R12A	Y762	13.2	1.83E-02	10.8	5.16E-04
Paxillin	Y88*	9.5	2.46E-06	2.7	3.09E-02
Paxillin	Y118*	6.6	7.72E-05	2.9	6.05E-03
PIKFYVE	Y1772*	12.2	2.49E-03	2.7	4.35E-02
Profilin 1	Y129*	3.2	6.25E-03	2.3	1.67E-02
PPP1CB	Y306*	99.9	1.27E-06	3.4	3.98E-03
ROCK2	Y722	3.0	2.56E-02	3.5	5.00E-03
TIAM1	Y384	24.2	1.09E-05	48.1	2.74E-02
VAV1	Y791	4.4	2.24E-06	6.0	3.82E-06
WASP	Y256*	53.1	2.07E-05	6.7	6.34E-05

¹ Ratio is calculated using average peak area of stimulated cells (Cold or CD3/4) divided by the average peak area of control cells

² Q value is calculated for multi-test control, based on p value resulted from unpaired student t test between replicated data of two groups of cells (stimulated vs. control)