Quantitative Analysis of Phosphotyrosine Signaling Networks Triggered by CD3 and CD28 Costimulation in Jurkat Cells

Ji-Eun Kim and Forest M. White

*J Immunol* 2006; 176:2833-2843; doi: 10.4049/jimmunol.176.5.2833

http://www.jimmunol.org/content/176/5/2833

**References**

This article cites 47 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/176/5/2833.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Quantitative Analysis of Phosphotyrosine Signaling Networks Triggered by CD3 and CD28 Costimulation in Jurkat Cells

Ji-Eun Kim and Forest M. White

The mechanism by which stimulation of coreceptors such as CD28 contributes to full activation of TCR signaling pathways has been intensively studied, yet quantitative measurement of costimulation effects on functional TCR signaling networks has been lacking. In this study, phosphotyrosine networks triggered by CD3, CD28, or CD3 and CD28 costimulation were analyzed by site-specific quantitative phosphoproteomics, resulting in identification of 101 tyrosine and 3 threonine phosphorylation sites and quantification of 87 sites across four cell states. As expected, CD3 stimulation induced phosphorylation of CD3 chains and upstream components of TCR pathways such as Zap70, while CD28 stimulation induced phosphorylation of CD28, Vav-1, and other adaptor proteins including downstream of tyrosine kinase 1, Grb2-associated protein 2 (Grap2), and Wiskott-Aldrich syndrome protein. CD3 and CD28 costimulation induced a complex response including increased threonine phosphorylation in the ERK1 and ERK2 activation loops and increased phosphorylation of selected tyrosine sites on ERK1/2, p38, phospholipase C-γ, Src homology 2 domain-containing transforming protein 1, Grap2, and Vav-1, potentiating T cell activation. Hierarchical clustering and self-organizing maps were used to identify modules of coregulated phosphorylation sites within the network. Quantitative information in our study suggests quantitative and qualitative contribution by costimulation of CD28 on CD3-stimulated TCR signaling networks via enhanced phosphorylation of phospholipase C-γ/Src homology 2 domain-containing transforming protein 1/Grap2/Vav-1 and their effects on downstream components including MAPKs. The Journal of Immunology, 2006, 176: 2833–2843.
focuses more on trans-activation of the TCR signaling network, with CD28 stimulation resulting in distinct signaling pathways leading to cytoskeletal rearrangement and mediation by key proteins such as P3K, Vav-1, and SLP-76 (7). In fact, cytoskeletal scaffolding following T cell stimulation is indispensable for T cell activation (11).

Studies to elucidate signaling pathways triggered by TCR stimulation or TCR and coreceptor stimulation have identified many of the components, their interactions, and protein phosphorylation sites in the TCR signaling network under a variety of conditions. These efforts have generated a fairly comprehensive map of the T cell signaling pathways, but quantitative functional analysis of T cell signaling components regulating responses to costimulation at the network level has been lacking. To elucidate the molecular mechanism of CD3/CD28 costimulation, a site-specific quantitative analysis of phosphotyrosine protein networks in CD-3, CD28-, and CD3/CD28-costimulated Jurkat cells was performed using a recently developed mass spectrometry (MS)-based phosphoproteomics method (12). Data analyzed in this study provide quantitative information on the role of both specific protein phosphorylation sites and functional modules effecting T cell activation following CD3 and CD28 costimulation.

Materials and Methods

Cell culture and TCR stimulation

Human Jurkat T cell line (provided by the Laufenburger Laboratory in the Massachusetts Institute of Technology Biological Engineering Division) was maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM t-glutamine, 0.1 mM non-essential amino acid solution, 10 mM HEPES buffer, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, and 55 mM 2-ME (Invitrogen Life Technologies) at 37°C under 5% CO2. Cells were grown in 75-cm² flasks with a starting density of 3×10⁶/ml followed by incubation for 5 min at 37°C. Cells were treated with CD28 stimulation resulting in distinct signaling pathways scaffolding following T cell stimulation is indispensable for T cell activation (11).

Cell lysates were desalted using Sep-Pak (Millipore) and lyophilized as described previously (12). Cells were lysed with 8 M urea supplemented with 1 M sodium orthovanadate (Sigma-Aldrich) and incubated for 20 min on ice. Fifty-microliter aliquots were used to measure protein concentration with the bicinchoninic acid protein assay (Pierce) for cross-linking. The reaction was stopped by cold PBS followed by centrifugation for subsequent lysis. Peptides were labeled with iTRAQ reagents (Applied Biosystems) according to the manufacturer’s instruction. Briefly, iTRAQ-labeled peptides derived from 1×10⁶ cells (200 µg of protein) were dissolved in 0.5 M triethylammonium bicarbonate and reacted for 1 h with two tubes of the iTRAQ reagents dissolved in ethanol (e.g., 200 µg of peptide from control sample was reacted with 2 tubes of iTRAQ-114). After incubation, samples labeled with four different iTRAQ reagents were combined and concentrated for subsequent immunoprecipitation with an anti-phosphotyrosine Ab mixture (12 µg each of pYtr100 from Cell Signaling Technology and PT66 from Sigma-Aldrich) bound to protein G-linked agarose beads in immunoprecipitation buffer (30 mM Tris, 30 mM NaCl, and 0.3% Nonidet P-40, pH 7.4) overnight at 4°C. The beads were washed with rinse buffer (100 mM Tris, 100 mM NaCl, and 0.3% Nonidet P-40, pH 7.4) three times and twice with immunoprecipitation buffer without Nonidet P-40. After the final rinse, immunoprecipitated peptides were eluted with 100 mM glycine (pH 2.5) for further immobilized metal affinity chromatography (IMAC)-based phosphopeptide enrichment.

Phosphopeptide enrichment using IMAC

IMAC columns were prepared as described elsewhere (13). Briefly, a 15-cm long (360 µm o.d.× 200 µm i.d.) microcapillary fused-silica column (Polymeric Technologies) was packed with POROS 20MC in water (Applied Biosystems) and washed with 100 nM EDTA (10 min at 10 µl/min). After rinsing with water to remove EDTA, the column was charged with 100 nM FeCl₃ for 10 min at 10 µl/min. Excess FeCl₃ was removed by rinsing with 0.1 M acetic acid, and the column was equilibrated with 0.1% trifluoroacetic acid. Immunoprecipitated peptides in 30% acetonitrile and 0.1% trifluoroacetic acid were loaded into the IMAC column at 1 µl/min. Remaining nonphosphorylated peptides were removed by washing with organic buffer comprised of 30% acetonitrile and 0.1% trifluoroacetic acid. The column was then equilibrated with 0.1% acetic acid (10 min at 10 µl/min) and phosphopeptides were eluted with 250 mM NaH₂PO₄ (pH 8.0) into a 10-cm long (360 µm o.d.× 100 µm i.d.) microcapillary fused silica precolumn packed with 10 µm C18 (YMC).

Liquid chromatography and tandem MS

After rinsing to remove excess phosphate buffer, the precolumn was connected to a 10-cm long (360 µm o.d.× 50 µm i.d.) microcapillary fused silica analytical column (packed with 5 µm C18 (YMC ODS-AQ)) with an integrated electropray ionization tip (1 µm). Peptides were eluted using a 120-min gradient: 0–13% B in 10 min, 13–50% B in 95 min, 42–60% B in 10 min, and 60–100% B in 5 min (B = 70% acetonitrile, 0.2 M acetic acid) and electrosprayed directly into a quadruple-time-of-flight mass spectrometer (QSTAR XL Pro; Applied Biosystems) with a flow rate <50 nl/min. The instrument was run in positive ion mode and cycled through information-dependent acquisition of a full-scan mass spectrum (m/z, 400-1500) (1.5 s), followed by four MS/MS scans (3 s each) sequentially on the four most abundant ions with two to five charges present in the full-scan mass spectrum.

Data analysis

All MS/MS spectra were searched against a human protein database (National Center for Biotechnology Information) with both MASCOT (Matrix Science) and ProQuant (Applied Biosystems) database searching algorithms. Search parameters contained a variable modification of +80 Da on serine, threonine, and tyrosine; variable neutral loss from phosphorylated serine and threonine amino acids, fixed modifications of carbamoyl at cysteine, and ITARQ at amino-terminal and lysine. Example spectra and process for sequence identification and quantification are shown in Fig. 1B. The majority of the fragment ions were automatically assigned to y- or b-type fragment ions in phosphorylated peptides. Phosphorylation sites and peptide sequence assignments contained in MASCOT or ProQuant search results were validated by manual confirmation from raw MS/MS data. Peak areas for each of four signature peaks (m/z; 114, 115, 116, 117, respectively) were obtained from ProQuant and corrected for isotopic overlap.

Bioinformatics and statistical analysis

Peak areas were normalized with values from the peak areas of nonphosphorylated peptides in supernatant of immunoprecipitation (analyzed separately by liquid chromatography (LC)-MS/MS). Each treatment was normalized against control sample to obtain an induced level of phosphorylation in CD3, CD28, and CD3/CD28 costimulation. Final normalized data sets (in Excel spreadsheets) were loaded into Spotfire and self-organizing maps and hierarchical analysis from Spotfire were used to cluster phosphorylation sites using built-in functions of the program. All MS/MS spectra were searched against a human protein database (National Center for Biotechnology Information) with both MASCOT (Matrix Science) and ProQuant (Applied Biosystems) database searching algorithms. Search parameters contained a variable modification of +80 Da on serine, threonine, and tyrosine; variable neutral loss from phosphorylated serine and threonine amino acids, fixed modifications of carbamoyl at cysteine, and ITARQ at amino-terminal and lysine. Example spectra and process for sequence identification and quantification are shown in Fig. 1B. The majority of the fragment ions were automatically assigned to y- or b-type fragment ions in phosphorylated peptides. Phosphorylation sites and peptide sequence assignments contained in MASCOT or ProQuant search results were validated by manual confirmation from raw MS/MS data. Peak areas for each of four signature peaks (m/z; 114, 115, 116, 117, respectively) were obtained from ProQuant and corrected for isotopic overlap.

Western blotting

Cell lysates were prepared by three freeze-thaw cycles in lysis buffer (20 mMol/L Tris-HCl, 150 mMol/L NaCl, 1 mMol/L EDTA, 1% Triton X-100, 0.5% Igepal, 2.5 mMol/L sodium PP₃, 1 mMol/L β-glycerophosphate, 1 mMol/L sodium orthovanadate, 1 mMol/L okadaic acid, 1 µg/ml leupeptin, 1 µg/ml bestatin, and 1 mMol/L phenylmethylsulfonyl fluoride) followed by centrifugation at 14,000 × g for 30 min. Seventy-five micrograms of total protein measured by the bicinchoninic acid protein assay (Pierce) was separated in 15% Tris-HCl gel (Bio-Rad) by SDS-PAGE and transferred onto a polyvinyliden difluoride membrane. The membrane was blocked with 5% nonfat milk in TBS with 0.1% Tween 20 for 1 h and then incubated with the indicated primary Ab in the same blocking buffer at 4°C
overnight. Rabbit anti-phospho Zap70 (Tyr292) Ab, mouse anti-phospho ERK (Tyr202) Ab (Santa Cruz Biotechnology), and mouse anti-phospho p44/42 MAPK (Thr202/Tyr204) Ab (Cell Signaling Technology) were used as primary Abs (1/1000 dilution). After washing with TBS and 0.1% Tween 20, the membrane was incubated with secondary anti-IgG Ab conjugated with HRP (1/100,000 dilution; Pierce) for 1 h. Blots were developed with supersignal West Femto substrate (Pierce). The membrane was reprobed with goat anti-actin Ab (Calbiochem) for normalization.

Results
Quantitative phosphoproteomics analysis of tyrosine phosphorylation in CD3-, CD28-, and CD3/CD28-costimulated Jurkat T cells resulted in the identification of 101 tyrosine and 3 threonine phosphorylation sites from 76 proteins, 87 of which were quantified across four cell states (full data set available at http://web.mit.edu/fwhitelelab/data). Briefly, protein extracts from stimulated Jurkat cells were enzymatically digested to peptides, which were chemically tagged before mixing. Tyrosine-phosphorylated peptides were isolated from the peptide mixture of four different cell states by immunoprecipitation, enriched by IMAC, and subjected to LC-MS/MS analysis (schematically represented in Fig. 1A). Following automated sequence assignment and quantification, manual validation was performed to eliminate quantification errors and to ensure accuracy of sequence and phosphorylation site assignment using raw data (an example is shown in Fig. 1B). Relative peak areas from iTRAQ marker ions (highlighted bottom left in Fig. 1B) present in the MS/MS analysis are representative of the level of phosphorylation on the given peptide from each of the four samples used for quantification.

Regulation of tyrosine phosphorylation in CD3 ITAMs and upstream kinases
ITAMs in CD3 and most of the upstream kinases identified in the data responded to CD3 stimulation but did not appear to play a role in the CD3/CD28 costimulation mechanism (other than PLC-γ). Tyrosine phosphorylation levels were quantified for every known ITAM of CD3δ, ε, γ, and ζ (Fig. 2A). Phosphorylation levels for each ITAM tyrosine site were increased 2- to 4-fold by CD3 stimulation, but were not further affected by CD3/CD28 costimulation (Fig. 2, B and C). Among protein kinases interacting with CD3 chains, phosphorylation sites in Lck and TCR Zap70 were identified and quantified (Fig. 3, B and C). Three tyrosine phosphorylation sites (Tyr192, Tyr394, and Tyr505) on Lck were identified and quantified. Of these, the tryptic peptide containing Tyr394 (LIED-Zap70, CD3 stimulation increased phosphorylation at Tyr292, a primary in vitro autophosphorylation site (14). Phosphorylation of Tyr292 appeared to be constitutive and did not change in response to any of the stimulations. Phosphorylation of Tyr505 was up-regulated 3-fold by CD3 stimulation, was unaffected by CD28 stimulation, and was down-regulated by CD3/CD28 costimulation. For Zap70, CD3 stimulation increased phosphorylation at Tyr202, a primary in vitro autophosphorylation site (14). Phosphorylation of this site was not further affected by CD3/CD28 costimulation (Fig. 3, B and C). In combination with phosphorylation of other downstream proteins, it seems that phosphorylation at Tyr202 in Zap70 corresponded with activation of the TCR pathway in this system, although it has been previously implicated in negative regulation using a mutated construct (15). Hierarchical clustering and self-organizing maps clustered Zap70 Tyr202 with CD3 ITAMs and PLC-γ (Fig. 4, A and B). Correspondingly, Zap70 has been implicated in transducing early T cell activation signaling from CD3 chains to downstream proteins such as PLC-γ (1–4). Finally, to compare with the quantitative MS data, Western blots were performed with an Ab recognizing Zap70 phosphorylated at Tyr202. Tyrosine phosphorylation levels measured by these two disparate analysis methods are in close agreement, as demonstrated in Fig. 3D.

Tyrosine phosphorylation sites in PLC-γ and two downstream proteins, protein kinase C-δ (PKC-δ) and calmodulin, were also quantified in this study (Fig. 3, B and C). Within PLC-γ, both Tyr771 and Tyr775 were detected and quantified; sequence information from a tandem MS experiment was sufficient to distinguish the peptides phosphorylated at two different tyrosine residues despite their proximity in the same peptide. Although both phosphorylation sites showed a similar level of increase in response to CD3

FIGURE 1. Experimental scheme. A, Experimental procedure. Briefly, Jurkat cells were serum starved (12 h) and stimulated (5 min) with either isotype control Ab, anti-human CD3 Ab, anti-human CD28 Ab, or anti-human CD3 Ab and anti-human CD28 Ab with goat anti-mouse IgG Ab for cross-linking. Following stimulation, cells were lysed, proteins were denatured and enzymatically digested to peptides, and peptides were chemically tagged before mixing. Tyrosine-phosphorylated peptides were isolated by immunoprecipitation with a mixture of anti-phosphotyrosine Abs, enriched by IMAC, and subjected to LC-MS/MS analysis. B, LC/tandem MS analysis and quantitative information from tandem MS spectrum. During LC-MS/MS analysis, peptides were acquired for ~2.5 h, during which time the instrument gathered >1000 MS spectra, each of which was queued to determine the four most abundant species, which were subsequently subjected to MS/MS analysis. Relative peak areas from iTRAQ marker ions present in the MS/MS analysis were quantified, representing the level of phosphorylation on the given peptide from each of the four samples. A peptide from CD3δ is presented as an example here: 1) TIC, Total ion chromatogram of peptide separation using LC; 2) MS, mass spectrum of selected time point; 3) MS/MS, tandem mass spectrum of selected peptide in a mass spectrum containing both sequence and quantification information (highlighted); and 4) signature peptide ions representing quantitative information in the low-mass range of tandem MS spectrum in B (graph 3). Each peak area is representative of the phosphorylation level of a tyrosine site in a peptide (DDAQpYSHLGGNWAR; CD3δ Y160) from control (m/z = 114), CD3 stimulated (m/z = 115), CD28 stimulated (m/z = 116), and both CD3/CD28 costimulated (m/z = 117).
stimulation (Fig. 3, B and C), phosphorylation of Tyr775 was further increased by costimulation (Table I). Studies on PKC-δ have focused on its phosphorylation at serine/threonine residues (16), its proapoptotic function (17), and antiapoptotic role (18) in diverse systems. CD3 stimulation induced a 2.5-fold increase in phosphorylation of PKC-δ/Tyr313, a level of which was not further affected by CD3/CD28 costimulation (Fig. 3, B and C). The role of PKC-δ, and particularly phosphorylation at Tyr313 in the unique sequence compared with other PKCs (RSDASSEPVGIPYQGFEK), has not been studied in T cell signaling. Both hierarchical clustering and self-organizing maps showed close connection of this site to CD3 chains, Shc, and p38 (Fig. 4, A and B). Relevantly, PKC-δ was reported to affect p38 MAPK in the IFN-γ-dependent pathway was also monitored; CD3 stimulation induced a 1.6-fold increase in phosphorylation at Tyr99 while CD3/CD28 costimulation did not further affect the phosphorylation level at this site (Fig. 3, B and C).

**Regulation of tyrosine phosphorylation in adaptor proteins**

Molecular adaptor/scaffold proteins play important roles by linking upstream kinases to downstream kinases or substrates (3, 20). LAT, Lck, and other adaptor proteins and kinases reside in glycolipid-enriched membrane microdomains/lipid rafts (GEM) under normal conditions. As shown schematically in Fig. 5A, other kinases and adaptor proteins are recruited to the GEM in response to TCR stimulation. Not surprisingly, quantitative changes in the phosphorylation level of many of the adaptor proteins were observed in response to CD3, CD28, or CD3/CD28 costimulation (Fig. 5, B and C (transmembrane adaptor proteins) and D (cytosolic adaptor proteins)). The most noticeable regulation is that CD3 stimulation increased phosphorylation of Tyr45 in Grb2-related adaptor protein 2 (Grap2, also known as Grb2-associated protein downstream of Shc (Gads)) by 2.4-fold and Tyr317 in Src homology 2 domain-containing transforming protein 1 (Shc) by 2.3-fold while CD3/CD28 costimulation further amplified their phosphorylation by 59 and 24%, respectively, suggesting their regulatory roles in response to CD3/CD28 costimulation (Table I). Grap2 shows similar SH3 binding specificity to Grb-2, but is expressed specifically in hemopoietic cells and known to interact with LAT, Sos, dynamin, sam68, and SLP-76, leading to Ag-stimulated endocytosis of TCR, cell proliferation, and cell cycling (3, 20). In both self-organizing maps and hierarchical clustering, Grap2 was clustered with CD3 chains, Zap70, and PLC-γ (Fig. 4, A).
A and B). These data, along with the highly amplified phosphorylation level in costimulation, suggest that Grap2 is regulated along with other upstream kinases during response to CD3 stimulation and moreover may be a critical regulator in CD3/CD28 costimulation. It is interesting to note that phosphorylation of Shc Tyr317 has been implicated in regulating the specific interaction between Shc and Grap2 (21), which may explain the coregulation of these proteins in our study. Tyr317 of p52 Shc was reported to be preferred by Lck whereas other sites such as Tyr239 and Tyr240 were phosphorylated by Syk and Zap70 in an overexpressed cell line system (22). However, our data showed better correlation between Tyr317 of Shc and Zap70 as compared with Lck (Figs. 3B and 6B). In hierarchical clustering and self-organizing maps, Shc Tyr317 phosphorylation was clustered closely with CD3-\(\gamma\) Tyr111, Tyr142, and Tyr153 and CD3-\(\gamma\) Tyr160 (also along with PKC-\(\delta\), p38, and Vav-1) (Fig. 4, A and B). In agreement with these results, Ravichandran et al. (23) have reported Shc binding to the phosphorylated CD3-\(\gamma\) chain.

Vav-1 has been highlighted in CD28 signaling and in response to CD3/CD28 costimulation (7, 8). Phosphorylation of Vav-1 was increased in response to both CD3 and CD28 stimulation by 1.9- and 2.7-fold for Tyr791 and 1.45- and 1.25-fold for Tyr844, respectively (Fig. 5, B and D). CD3/CD28 costimulation increased CD3-induced phosphorylation at Tyr791 by 38%, implying its involvement in costimulation (Table I). Phosphorylation at Tyr791 in the Wiskott-Aldrich syndrome protein (WASP) has been implicated in actin polymerization (24) and binding to Cdc42 (25). In our data set, WASP Tyr291 showed a pattern similar to Vav-1 Tyr844 (Fig. 5, B and D) with a slight increase (11%) in CD3/CD28 costimulation (Table I). WASP is also known to interact with intersectin-2 (26), an adaptor molecule involved in clathrin-coated vesicles and thereby in TCR endocytosis, which occurs a few minutes after TCR stimulation only if CD3 is fully activated (1). Hierarchical clustering shows a high correlation between tyrosine phosphorylation sites on WASP and Vav-1, which was also known to be involved in actin filamentation (7) (Fig. 4A). Self-organizing maps also cluster WASP Tyr291 with Vav-1 Tyr844 and calmodulin Tyr99 (Fig. 4B).

For the other members of the group of adaptor proteins, only minimal changes were seen for phosphorylation levels of SLP-76 associated protein (SLAP-130; also called Fyn-binding protein (Fyb-120/130)) and Src family-associated phosphoprotein 1 (Fig.

**FIGURE 4.** Clustering analysis of phosphotyrosine protein networks in stimulated Jurkat cells. A, Hierarchical clustering analysis. Three columns represent relative phosphorylation level in CD3-, CD28-, and CD3/CD28-costimulated Jurkat cells normalized against that in control cells. Yellow represents highly induced phosphorylation level and blue represents control level phosphorylation. Selected examples of clustered phosphorylation sites are highlighted. B, Clustering analysis using SOMs. Three columns represent the relative phosphorylation level in CD3-, CD28-, and CD3/CD28-costimulated Jurkat cells normalized against that in control cells. Optimal SOM architecture was a 3 × 4 matrix, as smaller matrices tended to cluster obviously dissimilar phosphorylation profiles.
Regulation of phosphorylation in MAPKs

ERK2 (p42) and ERK1 (p44) are MAPKs activated downstream of Ras (Fig. 6A). Although the level of the doubly phosphorylated (active) form of p42 and p44 increased dramatically (by 9.5- and 8.5-fold, respectively) in response to CD3 stimulation, this level decreased significantly (by 32 and 45.7%, respectively) in response to CD3/CD28 costimulation (Fig. 6, B and C). By comparison, the singly phosphorylated form of these proteins increased 5.8- and 5.7-fold, respectively, in response to CD3 stimulation (Fig. 6, B and C), whereas CD3/CD28 costimulation further amplified this response by 81.6 and 97.6%, respectively (Table I). In contrast to our MS data, Western blotting using an Ab recognizing the doubly phosphorylated form of p42/44 shows an apparent increase following CD3/CD28 costimulation, a pattern which was also seen when Western blots were probed with an Ab recognizing the singly phosphorylated form of these proteins (Fig. 6D). Based on the similarity between these Western blots, it is likely that the specificity of these Abs is insufficient to correctly quantify the relative phosphorylation levels of the two isoforms of these proteins. It is worth noting that the sequences of p42 and p44, while homologous, are divergent enough to readily distinguish each protein (and each phospho-isofrom) by MS (Fig. 6E). Quantitative analysis of singly phosphorylated p38 shows a similar pattern to singly phosphorylated p42/p44, albeit with a lower increase in absolute magnitude as compared to the other two MAPKs and no doubly phosphorylated form was detected (Fig. 6, B and C).

**Table I. Quantitative effects of CD3 and CD28 costimulation on CD3-stimulated phosphorylation**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phosphosite</th>
<th>Increase or Decrease by Costimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28</td>
<td>Y191*</td>
<td>150.7</td>
</tr>
<tr>
<td>p44Erk1</td>
<td>Y204*</td>
<td>97.6</td>
</tr>
<tr>
<td>p42Erk2</td>
<td>Y187*</td>
<td>84.6</td>
</tr>
<tr>
<td>Grap2/Gads</td>
<td>Y45</td>
<td>59.0</td>
</tr>
<tr>
<td>p38</td>
<td>Y182*</td>
<td>56.9</td>
</tr>
<tr>
<td>CD28</td>
<td>Y218*</td>
<td>50.4</td>
</tr>
<tr>
<td>Vav-1</td>
<td>Y781*</td>
<td>37.9</td>
</tr>
<tr>
<td>Cdc2</td>
<td>Y15*</td>
<td>33.8</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>Y775*</td>
<td>25.9</td>
</tr>
<tr>
<td>Shc</td>
<td>Y317*</td>
<td>24.1</td>
</tr>
<tr>
<td>Ribosomal protein P0</td>
<td>Y24</td>
<td>18.2</td>
</tr>
<tr>
<td>CD33</td>
<td>Y83*</td>
<td>15.2</td>
</tr>
<tr>
<td>Activating NKR</td>
<td>Y308</td>
<td>13.9</td>
</tr>
<tr>
<td>CD33</td>
<td>Y142*</td>
<td>13.8</td>
</tr>
<tr>
<td>Talin 1</td>
<td>Y70</td>
<td>12.5</td>
</tr>
<tr>
<td>WASP</td>
<td>Y291*</td>
<td>11.2</td>
</tr>
<tr>
<td>ITSN2</td>
<td>Y552</td>
<td>10.7</td>
</tr>
<tr>
<td>Amylo-1,6-glucosidase</td>
<td>Y584</td>
<td>-10.4</td>
</tr>
<tr>
<td>PECAM1</td>
<td>Y715</td>
<td>-11.0</td>
</tr>
<tr>
<td>Lck</td>
<td>Y393*</td>
<td>-13.5</td>
</tr>
<tr>
<td>PAG</td>
<td>Y417*</td>
<td>-18.5</td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1a</td>
<td>Y85</td>
<td>-19.1</td>
</tr>
<tr>
<td>GDP dissociation inhibitor 2 (GDI-2)</td>
<td>Y203</td>
<td>-19.2</td>
</tr>
<tr>
<td>PAG</td>
<td>Y387</td>
<td>-21.8</td>
</tr>
<tr>
<td>Lactate dehydrogenase A</td>
<td>Y10</td>
<td>-21.9</td>
</tr>
<tr>
<td>SHP2-interacting transmembrane adaptor protein</td>
<td>Y90*</td>
<td>-23.0</td>
</tr>
<tr>
<td>Sts-1</td>
<td>Y19</td>
<td>-25.4</td>
</tr>
<tr>
<td>PAG</td>
<td>Y341*</td>
<td>-31.9</td>
</tr>
<tr>
<td>p42ERK2</td>
<td>T185+ Y187*</td>
<td>-32.0</td>
</tr>
<tr>
<td>Dok-1</td>
<td>Y362*</td>
<td>-32.6</td>
</tr>
<tr>
<td>Dok-1</td>
<td>Y296*</td>
<td>-36.4</td>
</tr>
<tr>
<td>CD5</td>
<td>Y453*</td>
<td>-39.3</td>
</tr>
<tr>
<td>Cdc2</td>
<td>T14* Y15*</td>
<td>-43.5</td>
</tr>
<tr>
<td>p44Erk1</td>
<td>T202* Y204*</td>
<td>-45.7</td>
</tr>
<tr>
<td>Lck</td>
<td>Y504*</td>
<td>-70.1</td>
</tr>
</tbody>
</table>

* Previously reported phosphorylation site.

5, B and D). Both downstream of tyrosine kinase 1 (Dok-1) and phosphophosphate associated with glycosphingolipid-enriched microdomains (PAG) were constitutively phosphorylated as previously reported (27, 28). Phosphorylation levels at multiple sites in Dok-1 and PAG were increased slightly following CD3 stimulation; this effect was largely abolished by CD3/CD28 costimulation (Fig. 5, B–D), similar to the pattern displayed by Lck Tyr505.

**Regulation of tyrosine phosphorylation in components of CD28 signaling pathways**

Whether CD28 contributes to TCR signaling pathways qualitatively or quantitatively remains a question in T cell activation. Identification of components regulated distinctively in response to CD28 stimulation was therefore of interest in this study. Several CD28 tyrosine phosphorylation sites have been described in the literature. Tyr191 in the YMMN motif of the CD28 cytoplasmic tail (7, 8) has been shown to recruit PI3K and negatively regulate HIV-1 transcription (29). Tyr218 in CD28 was also shown to be involved in Vav-1 tyrosine phosphorylation, Rac1 activity (30) and, along with Tyr206 and Tyr209, in IL-2 secretion (31). Not surprisingly, both CD28 phosphorylation and CD3/CD28 costimulation resulted in increased phosphorylation levels of CD28 Tyr191 and Tyr218. Interestingly, in our data set, phosphorylation levels of CD28 Tyr191 and Tyr218 were increased by CD3 stimulation (Fig. 7, B and C), which suggests recruitment of CD28 into lipid rafts, potentially resulting in its phosphorylation by shared protein tyrosine kinases activated during CD3 stimulation. The phosphorylation level of PI3K p85 Tyr467 and glycogen synthase kinase 3B (Gsk3) Tyr279 were unaffected by CD3, CD28, or CD3/CD28 costimulation. However, CD3 stimulation induced a 23% increase ($p < 0.001$) in phosphorylation at Tyr12 of IL-2-inducible T cell kinase (Itk). Phosphorylation of this site has previously been linked to Lck activity and is reported to activate Itk (32) (Fig. 7, B and C). In Jurkat cells, PI3K and Itk are known to be constitutively active due to the high level of phosphatidylinositol(3,4,5)triphosphate from phosphatase and tension analog deficiency (33–35).

**Clustering of identified phosphorylation sites in CD3, CD28, and CD3/CD28 costimulation**

To identify coregulated phosphorylation sites, the data were clustered using both hierarchical clustering (Fig. 4A) and self-organizing maps (Fig. 4B). In hierarchical clustering, sites most closely coregulated are grouped together and a distance metric is provided to estimate the variance between clusters of grouped sites. This method was able to successfully cluster phosphorylation sites expected to be coregulated such as the singly and doubly phosphorylated forms of ERK1 and ERK2. A different cluster contained many of the phosphorylation sites on CD3 chains along with sites on Grap2, PLC-γ, and Zap70, while a closely related cluster contained the remainder of the CD3 tyrosine phosphorylation sites, p38, Sts-1, PKC-δ, and Shc (Fig. 4A). Self-organizing maps (SOMs) have been applied to cluster self-similar temporal gene...
proteins such as Grap2, Dok-1, and WASP and kinases such as Vav-1 and smaller increases in phosphorylation levels of adaptor proteins. By comparison, CD28 stimulation resulted in a large increase in phosphorylation of CD28 and Vav-1 beyond levels detected following either CD3 or CD28 stimulation, and a few adaptor proteins (Grap2, Shc, and Vav-1) beyond levels detected following either CD3 or CD28 stimulation (Fig. 8). These results indicate that the response to CD3/CD28 costimulation is largely due to the increase or decrease of phosphorylation levels on sites which are also utilized in response to CD3 stimulation, in agreement with the quantitative mechanism of T cell activation in response to CD3/CD28 costimulation. More specifically, data generated in this study suggest that the mechanism of costimulation to complete T cell activation may be enhanced tyrosine phosphorylation of PLC-γ/Shc/Grap2/Vav-1 leading to enhanced activation of downstream MAPKs and transactivation activities of AP-1 complex on IL-2 expression (1), which has been used as a marker for T cell activation in Jurkat cells for >20 years (35). Interestingly, amplification of tyrosine phosphorylation in ERK1/2, and PKC-δ (Fig. 8B). CD3/CD28 costimulation amplified tyrosine phosphorylation of CD28, MAPKs (p42 (ERK2), p44 (ERK1), p38), PLC-γ, and a few adaptor proteins (Grap2, Shc, and Vav-1) beyond levels detected following either CD3 or CD28 stimulation (Fig. 8C and Table I). These results indicate that the response to CD3/CD28 costimulation is largely due to the increase or decrease of phosphorylation levels on sites which are also utilized in response to CD3 stimulation, in agreement with the quantitative mechanism of T cell activation in response to CD3/CD28 costimulation. More specifically, data generated in this study suggest that the mechanism of costimulation to complete T cell activation may be enhanced tyrosine phosphorylation of PLC-γ/Shc/Grap2/Vav-1 leading to enhanced activation of downstream MAPKs and transactivation activities of AP-1 complex on IL-2 expression (1), which has been used as a marker for T cell activation in Jurkat cells for >20 years (35). Interestingly, amplification of tyrosine phosphorylation in ERK1/2, p38, and Cdc2 by CD3/CD28 costimulation was the most noticeable change in this study and was accompanied by a decrease in threonine phosphorylation levels on the same peptides of these downstream components. Amplification of the singly (threonine) phosphorylated form might be explained simply by phosphatase activity leading to the conversion of the doubly (threonine and tyrosine) phosphorylated form back to the singly phosphorylated form. Among the known phosphatases affecting these proteins, we did not detect MAPK phosphatases or Cdc25C phosphatase activity leading to the conversion of the doubly (threonine and tyrosine) phosphorylated form back to the singly phosphorylated form. Among the known phosphatases affecting these proteins, we did not detect MAPK phosphatases or Cdc25C phosphatase activity leading to the conversion of the doubly (threonine and tyrosine) phosphorylated form back to the singly phosphorylated form.
FIGURE 6. Regulation of phosphorylation sites in MAPKs in response to T cell stimulation. A, MAPK pathway. B, Quantification results of specific phosphorylation sites in MAPKs in response to CD3, CD28, and CD3/CD28 costimulation. Data represent mean of results obtained from three experiments; *, Previously reported phosphorylation site. C, Graphical representation of quantification data in B. D, Western blotting for specific phosphorylation sites in p42/44 MAPK using Abs recognizing either the doubly phosphorylated form of p44/42 (upper blot) or singly phosphorylated form of p44/42 (lower blot). Data are representative of three experiments. Actin was used as a loading control (Ctrl). E, Comparison of quantification of doubly and singly phosphorylated forms of both p42 ERK2 and p44 ERK1. MS/MS spectra for each peptide (graphs 1–4) contain y- and b-type ions which enable sequence and phosphorylation site assignment, while peak areas for iTRAQ marker ions enable quantification of phosphorylation from each sample. Solid arrows and molecular mass of each precursor peptide highlight evidence for the correct sequence while dashed arrows highlight evidence for the phospho-isoform of p42 ERK2 and p44 ERK1.

phosphorylated at both Thr14 and Tyr15, resulting in entry into cycle entry (39), and, at the G2-M boundary, Cdc2 becomes de-threonine phosphorylation. One outcome of T cell activation is cell entry by enhancing singly phosphorylated Cdc2 via decreased phatase could occur during CD3/CD28 costimulation, potentially it is likely that activation of an alternate threonine-specific phosphatase may answer these questions, but network level quantification for low abundant phosphoserine/threonine proteins remains a challenging task due to the complexity and dynamic range of the serine/threonine phosphoproteome (41).

As suggested above, phosphatase activation may explain the decrease of doubly phosphorylated Cdc2 peptide (IGEGpTpYGVVYK) by 33.8% on costimulation and the increase of the singly phosphorylated peptide (IGEGpYGVVYK) by 43.5%. However, it cannot adequately justify the results for ERK1 and ERK2, as the increase in tyrosine phosphorylation (97.6 and 81.6%, respectively) was much greater than could be accounted for the corresponding decrease in threonine phosphorylation (45.7 and 32%, respectively, Table I). Increased tyrosine phosphorylation of MAPKs after CD3/CD28 costimulation is therefore indicative of enhanced potentiation (potential future activation) of these proteins, a state that may be evident in analyses of later time points for these signaling networks. In sum, our study of CD3 and CD28 costimulation mechanism demonstrates an amplified phosphorylation level of upstream proteins such as CD28, PLC-γ, Shc, and Grap2, likely involvement of phosphatases in selected parts of the network, and enhanced tyrosine phosphorylation of MAPKs, suggesting an increase in network potentiation along with amplified signaling processes for complete T cell activation.

Along with identification of specific components and their interactions in signaling pathways, several efforts to understand the costimulatory mechanism in T cell signaling pathways at the systems level have recently started. For example, phosphorylation site-specific Ab-dependent protein microarray has been shown to be a sensitive tool to monitor alterations in phosphorylation levels of the known components in TCR signaling pathways (42). Compared with protein microarrays, our MS-based phosphoproteomics method combined with tyrosine peptide-specific immunoprecipitation (43) provides a complementary approach to understanding functional networks. Specifically, our method provides an Ab-independent tool for uncovering previously uncharacterized phosphorylation sites, as demonstrated by the identification of 43 previously uncharacterized tyrosine phosphorylation sites, 32 of which were quantified. Additionally, our MS-based method enables network level analysis while maintaining high-resolution quantitative information on phosphorylation site-specific regulation. For instance, by tandem MS it was possible to distinguish and quantify phosphorylation levels of singly and doubly phosphorylated ERK1 and ERK2 individually (Fig. 6E), while commercially available Abs cannot distinguish the two different MAPKs and appear to show cross-reactivity between singly and doubly phosphorylated forms of the kinase (Fig. 6D). It is also worth noting that sequence information from tandem MS was sufficient to distinguish and quantify phosphorylation at Tyr771 and Tyr775 in a peptide from PLC-γ, respectively (Fig. 3, B and C). In this study, we have demonstrated a method enabling both identification and quantification of tyrosine-phosphorylated proteins at the network level from biologically relevant, TCR- and CD28-stimulated Jurkat cells. Several other groups have previously applied MS to investigate T cell signaling components. Rush et al. reported a list of tyrosine-phosphorylated proteins (43) and Tao et al. (44) adopted dendrimer chemistry to enrich phosphorylated peptides from pervanadate-treated Jurkat cells. Not surprisingly, pervanadate treatment dramatically enhances levels of protein tyrosine phosphorylation compared with anti-CD3 Ab treatment, which can be easily detected by anti-phosphotyrosine Western blotting (data not shown) and has been compared previously (45). Although pervanadate-stimulated cells can be useful, the strong activity of this compound and the lack of regulatory information in biological systems limit the utility of these cells to
development of methodology for improved identification of phosphoproteins. Other laboratories have used MS to investigate T cell signaling in response to particular stimuli; recently Salomon et al. (46) reported quantitative regulation of CD3-ζ in CD3/CD4-stimulated Jurkat cells using an external control and Zheng et al. reported a ΔD/ΔD-based isotope-based quantitative methodology for IFN-α-treated Jurkat cells (47). Compared with these reports, we were able to scale down the number of cells 30- to 200-fold (from 0.3–2 × 10^5 to 1 × 10^4) per treatment and compare quantitative changes in more biologically relevant samples without an external control, thereby reducing errors associated with run-to-run variability. Isobaric-stable isotope tags (48) used in this method also provide several advantages, including coelution of the same peptide from all four different treatments and accumulation of MS signals in MS mode and MS/MS mode, resulting in higher sequence coverage and improved identification of low-abundant peptides.

Our site-specific quantitative phosphoproteomics approach identified networks of tyrosine-phosphorylated proteins triggered and regulated by CD3, CD28, and CD3/CD28 costimulation in Jurkat cells. Quantitative information suggests that CD3/CD28 costimulation may further activate the TCR signaling pathway mediated by enhanced phosphorylation of PLC-γ/SHC/Grap2/Vav-1 and their effects on downstream components including MAPKs. Although Vav-1 and WASP seem to be primarily activated in CD28 stimulation, potentially explaining a qualitative effect of costimulation leading to cytoskeletal rearrangement, enhanced phosphorylation of other upstream regulators suggests more quantitative than qualitative contribution by CD28 stimulation on CD3-stimulated TCR pathways. Application of bioinformatics tools to cluster coregulated phosphorylation events revealed and confirmed the presence of modules within tyrosine-phosphorylated signaling networks, such as the relationship between CD3 chains, tyrosine protein kinases, and adaptors. Our overall approach combining mass spectrometric analysis of tyrosine-phosphorylated peptides with clustering tools successfully identified, quantified, and delineated tyrosine-phosphorylated protein networks, highlighting at a mass spectrometry and its application to Saccharomyces cerevisiae. Nat. Bio- technol. 20: 301–305.


Acknowledgments

We thank D. Mathis and C. Benoist at the Joslin Diabetes Center and members of the White laboratory at Massachusetts Institute of Technology for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

References


Acknowledgments

We thank D. Mathis and C. Benoist at the Joslin Diabetes Center and members of the White laboratory at Massachusetts Institute of Technology for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

References


