

Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry

W Andy Tao^{1,2}, Bernd Wollscheid², Robert O'Brien², Jimmy K Eng², Xiao-jun Li², Bernd Bodenmiller³, Julian D Watts², Leroy Hood² & Ruedi Aebersold²⁻⁴

We present a robust and general method for the identification and relative quantification of phosphorylation sites in complex protein mixtures. It is based on a new chemical derivatization strategy using a dendrimer as a soluble polymer support and tandem mass spectrometry (MS/MS). In a single step, phosphorylated peptides are covalently conjugated to a dendrimer in a reaction catalyzed by carbodiimide and imidazole. Modified phosphopeptides are released from the dendrimer via acid hydrolysis and analyzed by MS/MS. When coupled with an initial antiphosphotyrosine protein immunoprecipitation step and stable-isotope labeling, in a single experiment, we identified all known tyrosine phosphorylation sites within the immunoreceptor tyrosine-based activation motifs (ITAM) of the T-cell receptor (TCR) CD3 chains, and previously unknown phosphorylation sites on total 97 tyrosine phosphoproteins and their interacting partners in human T cells. The dynamic changes in phosphorylation were quantified in these proteins.

Reversible protein phosphorylation has a vital role in regulating many complex biological processes such as cellular growth, division and signaling¹. Furthermore, the regulation of phosphorylation-dependent cell signaling has a major role in human diseases, most notably cancer². Because of this, there has been considerable interest in methodologies for the identification of regulatory sites of protein phosphorylation. More recently, advances in mass spectrometry (MS)-based proteomics have driven the search for reliable proteomic approaches for large-scale phosphorylation screening methods that include the identification of protein phosphorylation sites as well as the quantification of the phosphorylation changes at individual sites³⁻⁵. Whereas MS has emerged as a powerful tool for rapid characterization of proteins and for the analysis of other co- and post-translational modifications, a general MS-based proteomic approach for phosphorylation analysis has remained somewhat elusive⁶.

Phosphorylated proteins and, in particular, the dynamically phosphorylated forms of signaling proteins, are often of low abundance. In contrast to the identification of phosphorylated proteins in general, the identification of the phosphorylation sites within these phosphoproteins is far more difficult. To identify the sites of phosphorylation, it is essential to have an efficient strategy for the enrichment of actual phosphopeptides. Several approaches have been explored to date for the selective isolation of phosphopeptides; the most notable of these are either affinity- or chemical derivatization-based. Phosphotyrosine antibodies have been used successfully for the isolation of phosphoproteins⁷⁻¹⁰ and, more recently, tyrosine phosphopeptides¹¹. Immobilized metal ion affinity chromatography has been extensively used for the isolation of phosphopeptides, and steady steps have been taken to improve its specificity^{12,13}. Still, the method appears to be highly dependent on the type of resin and pH condition used for binding and elution, and favors peptides with multiple phosphorylation sites¹⁴. Chemical modifications of phosphate groups, to facilitate the isolation of phosphopeptides, are most promising for improving the specificity, but typically involve several derivatization steps, and to date have had limited applications for the analysis of phosphopeptides within complex mixtures^{15,16}. It is therefore apparent that a new method for phosphorylation analysis, in particular quantitative measurements, fulfills an urgent need.

Here we report a strategy for the enrichment and identification of phosphopeptides from complex mixtures based on a new chemical procedure that also allows for quantitative measurements of phosphorylation changes. By using a commonly-used derivatization of phosphate to phosphoramidate groups, we covalently coupled phosphorylated peptides to a synthetic polyamine (dendrimer) in a single step. The covalently immobilized phosphopeptides were then readily recovered via acid hydrolysis, allowing for their subsequent characterization via liquid chromatography (LC)-MS/MS. The method is generally applicable to the analysis of serine/threonine/tyrosine phosphorylation in complex protein mixtures. We also

¹The Bindley Bioscience Center and Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907, USA. ²Institute for Systems Biology, 1441 North 34th Street, Seattle, Washington 98103, USA. ³Institute for Molecular Systems Biology, Federal Institute of Technology, Campus Hoengerberg, HPT E78, Wolfgang Pauli Strasse 16, CH-8093, Zurich, Switzerland. ⁴Faculty of Natural Sciences, University of Zurich, Winterthurerstr. 190, 8057, Zurich, Switzerland. Correspondence should be addressed to R.A. (aebersold@imsb.biol.ethz.ch).



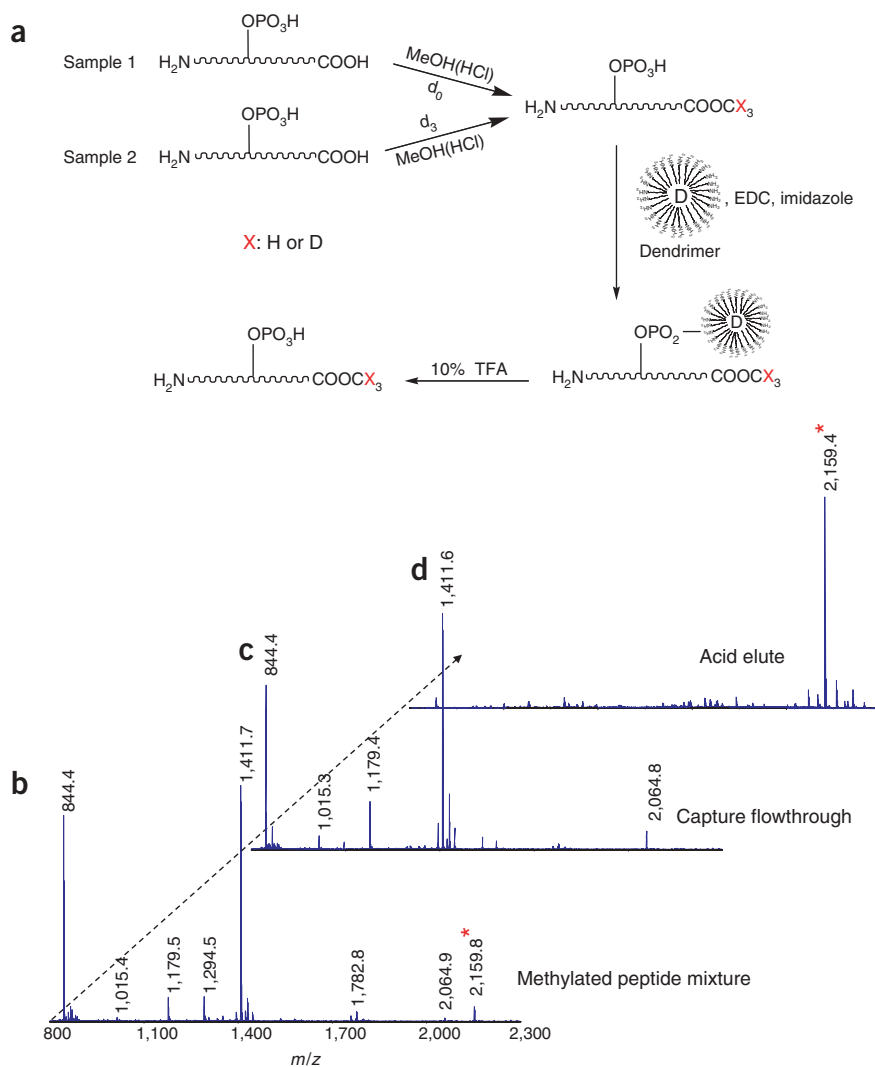


Figure 1 | Isolation and tagging of phosphopeptides, and validation with β -casein digests. **(a)** Schematic illustration of a three-step procedure to chemically isolate phosphopeptides. Peptides from samples 1 and 2 are d_0 - and d_3 -methylated, respectively. Methylated peptides are combined and subjected to a dendrimer conjugation catalyzed by EDC and imidazole. Phosphopeptides are captured on the dendrimer, whereas nonphosphopeptides are removed by extensive washing steps. Finally methylated phosphopeptides are released from the dendrimer via acid hydrolysis and analyzed by MS/MS. **(b–d)** Isolation of phosphopeptide FQS*EEQQTEDELQDK from β -casein digests according to the procedure in **a**. Spectra were obtained using a MALDI-TOF/TOF mass spectrometer. Starting material for the isolation is 100 pmol β -casein digests. For each step, 0.5 pmol of sample was used for mass spectrometric analyses. The red star in the spectra indicates a phosphopeptide. Other ions from β -casein digests were labeled. In **d**, the side product of single demethylation of 2,159.4 was observed (<5%). Other trace peaks are salt or matrix ions and unknown contaminations. No other ions from β -casein digests were detected.

amino groups and allows for the coupling of phosphopeptides directly to the dendrimer without extra purification steps. Covalently bound phosphopeptides are readily isolated from nonphosphopeptides using size selective methods such as a simple membrane-based filter device. In the final step, phosphopeptides are detached from the dendrimer through a brief acid hydrolysis of the phosphoramidate bonds and isolated using the same membrane-based filter device.

demonstrate the identification and quantification of the phosphorylation sites within peptides from purified synthetic phosphoproteins to biologically interesting phosphotyrosine kinase (PTK) substrates in complex human T-cell lysates.

RESULTS

Strategy for the labeling and enrichment of phosphopeptides

The overall strategy for labeling and isolating phosphopeptides is a three-step procedure (Fig. 1a). In the first step, mixtures of peptides are converted to the corresponding methyl esters. This step protects carboxylate groups from further reactions during subsequent steps. This also introduces stable isotopes for quantitative analysis. Peptide mixtures prepared from two cellular states are differentially labeled via the methyl esterification with either methanol (CH_3OH) or deuterated methanol (CD_3OD)^{12,17}. In the second step, aimed at tagging and isolating phosphopeptides, the methylated peptides are combined and subjected to a one-pot reaction in the presence of carbodiimide (that is, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide; EDC), imidazole and a dendrimer. Phosphate groups are readily activated using carbodiimide and imidazole and react with excess amines on the dendrimer to form phosphoramidate bonds^{15,18}. The one-pot reaction eliminates the need to protect

Isolation and quantification of standard phosphopeptides

We applied the strategy first to the isolation of phosphopeptides from the phosphoprotein β -casein. Tryptic digestion of β -casein generated several peptides. Before applying the enrichment strategy, the intensity of the methylated phosphoserine peptide FQS*EEQQTEDELQDK (2,159.8 *m/z*; * indicates a phosphorylated residue) was less than 10% of that of the base peak in a matrix-assisted laser desorption ionisation (MALDI) time-of-flight (ToF)/ToF mass spectrum (Fig. 1b). Upon capturing of the methylated phosphopeptide on a dendrimer, only the nonphosphopeptides were detected in the flowthrough (Fig. 1c). Upon the release of the phosphoserine peptide by a brief acid treatment, the phosphorylated peptide was detected as the most prominent peak in the mass spectrum, with little contamination from nonphosphopeptides or other contaminants (Fig. 1d). The overall sample recovery efficiency was examined by isotopic labeling of 10 pmol of the tryptic peptides from β -casein by methyl- d_0 and - d_3 esterification. Methyl- d_0 -esterified peptides were subjected to an enrichment step and the recovered methyl- d_0 phosphopeptide was added into the original methyl- d_3 peptide mixtures. The phosphopeptide recovery yield efficiency was determined by measuring the relative abundance of methyl- d_0 and - d_3 phosphopeptides in the mass spectrum,

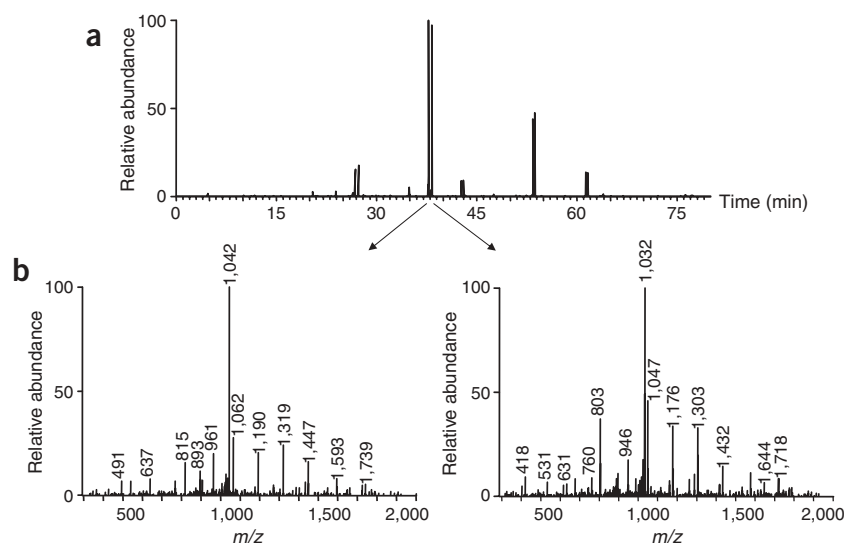


Figure 2 | Quantitation of phosphopeptides in standard protein mixtures. **(a)** LC-MS ion chromatogram of the phosphopeptides with neutral loss of 49 Da isolated from the six-protein mixture. **(b)** Product ion spectra of m/z 1,091 (left) and 1,081 (right). Similar fragmentation patterns of two precursors indicate the two peptides have the same sequence. The m/z differences in fragment ions in two spectra are due to differential isotopic labeling.

with a final yield of >35% of the starting material (data not shown).

To evaluate the above strategy for its ability to quantify the extent of phosphorylation in complex mixtures, two mixtures consisting of equimolar quantities of ovalbumin, BSA, β -lactoglobulin, lysozyme, β -casein and apomyoglobin (10 pmol each) were prepared and analyzed. Each protein mixture was digested and either methyl- d_0 or methyl- d_3 esterified. Esterified tryptic peptides were pooled and treated as depicted in **Figure 1**. Subsequently, 10% of the isolated and tagged phosphopeptides (the equivalent of phosphopeptides from 1 pmol each of protein mixture) were quantified and sequenced in a single microcapillary LC-MS experiment on an electrospray ionization (ESI) ion trap mass spectrometer. Serine/threonine phosphopeptides lose phosphoric acid (corresponding to a neutral loss event of 98 Da for singly charged ions or 49 Da for doubly charged ions in mass spectra) readily upon a collision-induced dissociation process in the ion-trap mass spectrometer. Thus, tracing ions with a dominant neutral loss of 49 Da in MS/MS spectra strongly suggests that the peptide is a doubly charged serine or threonine phosphopeptide (**Fig. 2**). In the above experiment, only a few ions with a dominant neutral loss of 49 Da were detected, indicating the specificity of the method. These phosphopeptides were from the phosphoproteins β -casein and ovalbumin. For example, a single pair of doubly-charged phosphopeptides of m/z 1,081 and 1,091 was identified via a SEQUEST software database search as the serine phosphopeptide FQS*EEQQTEDELQDK from β -casein with H- and D-methyl esterification, respectively. The peptide has a total of seven carboxylic groups, and after methyl- d_0 and $-d_3$ esterification, the mass difference between heavy and light isoforms is 21 Da (10.5 Da for doubly charged ions). Quantification of these two differentially labeled peptides was achieved by reconstructing and integrating the contour of the two respective ion chromatograms. The ratio (light:heavy) was determined to be 0.89. To calculate correct ratios,

it is important to notice here that deuterated (heavy) peptides eluted slightly earlier than nondeuterated (light) peptides on the capillary reverse phase liquid chromatography column owing to the incorporation of the isotopes. Therefore, accurate quantification required integration of the peak areas¹⁹, a task that is easily accomplished by quantification tools developed for quantitative proteomics²⁰.

The sensitivity of the method for quantitative analysis was tested with a peptide mixture in which a highly abundant background (500 pmol of BSA digest) was spiked with 1 pmol of a single phosphopeptide (phosphorylated angiotensin II, DRVY*IHPF). The peptide mixture was divided equally, and each sample was either methyl- d_0 or methyl- d_3 esterified. Samples were then combined and subjected to the enrichment steps, as schematically illustrated in **Figure 1a**. Aliquots of recovered phosphopeptides (as methyl- d_0 and $-d_3$ esters) were analyzed by μ LC-MS/MS to monitor the sensitivity of the method and

its quantitative nature at low concentration. Using a sensitive quadrupole linear ion trap mass spectrometer (LTQ), the method allows detection of phosphopeptides in complex mixtures down to the level of 10 fmol of phosphopeptides with expected abundance ratio (data not shown), demonstrating that the method has the capability to analyze low-abundance signaling molecules in biological samples.

Analyses of tyrosine phosphorylation sites in human T cells

Next, we applied the strategy to identify and assess the phosphorylation sites of kinase substrates in activated human T cells. Stimulation of T cells via cell-surface receptors triggers the activation of the TCR and then activates intracellular networks of effector molecules^{21,22}. Dynamic serine/threonine/tyrosine phosphorylation and dephosphorylation of signaling intermediates by kinases and phosphatases, respectively, is critical for the regulation of the T-cell signaling network²³. The phosphorylation status of signaling molecules modulates dynamic protein-protein interactions and allows for the integration of extracellular signals into various T-cell responses. To fully understand T-cell responses, it is necessary to pinpoint temporal phosphorylation and dephosphorylation events in the course of the response of T cells to various stimuli. We therefore used T cells as a model system to test the capability of our approach to measure phosphorylation changes. As we were especially interested in identifying tyrosine phosphorylation sites, we used the inhibitor pervanadate to elevate PTK substrate levels.

We treated aliquots of Jurkat T cells with pervanadate for 2 or 10 minutes, before lysing them. Because tyrosine phosphorylation sites are less frequent compared to serine/threonine phosphorylation sites, we coupled an immuno-affinity step to our dendrimer-based phosphopeptide enrichment method to enrich for tyrosine-phosphorylated proteins. Phosphotyrosine antibodies were used to first immuno-enrich tyrosine phosphoproteins

from the lysate. Methyl- d_0 and $-d_3$ esters of tryptic peptides generated from this enriched protein population were then combined, and phosphopeptides were isolated using the dendrimer-based derivatization method followed by microcapillary reverse-phase LC-MS/MS analysis.

This tandem purification method allowed for the identification and quantification of a total of 97 tyrosine phosphoproteins and their interacting partners with 75 tyrosine phosphorylation sites, as well as 80 serine and threonine phosphorylation sites in these proteins. Confidence in these identifications was assigned using

Table 1 | Selected phosphorylated peptides from immuno-purified Jurkat lysates

IPI number	Protein name	Peptide sequence ^a	Ratio ^b	Reference
IPI0000861	LASP1	H.HIPTSAPVY*QQPQQPVAQSYGGYK.E	1.62 ± 0.34	36
IPI00003479	ERK1	R.VADPDHDHTGFLTEY*VATR.W	0.72 ± 0.10	37
IPI00004407	SIT	K.Y*SEVVDSEPK.S	1.09 ± 0.16	8
IPI00004407	SIT	R.LS*QDPEPDQQDPTLGGPAR.A	0.74 ± 0.06	
IPI00004407	SIT	R.SGES*VEEVPLYGNLHYLQT*GR.L	2.61 ± 0.77	
IPI00015287	DOC1	K.SHNSALY*SQVQK-.S	0.36 ± 0.10	38
IPI00015287	DOC1	R.VKEEGY*ELPYNPATDDY*AVPPPR.S	1.01 ± 0.05	39
IPI00015287	DOC1	K.EDPIY*DEPEGLAPVPPQGLY*DLPR.E	1.25 ± 0.31	39
IPI00015287	DOC1	R.ADS*HEGEVAEGK.L	1.20 ± 0.16	
IPI00022602	DOC2	R.GQEGEY*AVPFDAVAR-.S	0.47 ± 0.04	38
IPI00022934	CD3δ	R.DDAQY*SHLGGNWAR.N	1.99 ± 0.36	38
IPI00022934	CD3δ	R.DRDDAQY*SHLGGNWAR-.N	1.55 ± 0.09	
IPI00022934	CD3δ	R.NDQVY*QPLR.D	1.83 ± 0.38	38
IPI00012923	CD3ε	R.DLY*SGLNQR.R	1.74 ± 0.28	40
IPI00012923	CD3ε	K.ERPPPVPNPDY*EPIR.K	1.56 ± 0.13	
IPI00016020	CD3γ	R.EDDQY*SHLQGNQLR.R	1.23 ± 0.12	
IPI00016020	CD3γ	K.QTLLPNDQLY*QPLK.D	1.70 ± 0.23	
IPI00218634	CD3ζ	K.DTY*DALHMQUALPPR.-	0.92 ± 0.15	9
IPI00218634	CD3ζ	K.GHDGLY*QGLSTATK.D	1.05 ± 0.32	30
IPI00218634	CD3ζ	R.KNPQEGLY*NELQK-.D	0.99 ± 0.11	30
IPI00218634	CD3ζ	K.MAEAY*SEIGMK.G	1.40 ± 0.08	30
IPI00218634	CD3ζ	K.NPQEGLY*NELQK.D	1.08 ± 0.12	30
IPI00218634	CD3ζ	R.REEY*DVLDK.R	1.24 ± 0.29	30
IPI00218634	CD3ζ	R.SADAPAYQQGQNLQY*NELNLGR-.R	1.59 ± 0.15	8
IPI00218634	CD3ζ	R.SADAPAY*QQGQNLQY*NELNLGR.R	2.10 ± 0.23	8
IPI00021076	Splice isoform of Plakophilin 4	R.SAVSPDLHITPIY*EGR.T	1.00 ± 0.10	
IPI00021076	Splice isoform of Plakophilin 4	R.SSY*ASQHSQGLQDLR.S	4.13 ± 1.14	
IPI00022339	CD28	R.LLHSDY*MNMTPR.R	2.53 ± 0.41	41
IPI00022339	CD28	K.HYQPY*APPR.D	1.08 ± 0.81	41
IPI00023704	LPP	R.NSDPTY*GQQGHPNTWK.R	3.02 ± 0.70	
IPI00023704	LPP	R.YYEGYY*AAGPGYGGGR.N	1.85 ± 0.38	8
IPI00026156	HS1	K.SAVGHEY*VAEVEK.H	1.63 ± 0.19	
IPI00026156	HS1	R.S*PEAPQPVIAMEEPAVPAPLPK.K	0.50 ± 0.21	
IPI00030851	Sec24B	N.TVNQQPGAQQLY*SR.G	1.12 ± 0.21	
IPI00030851	Sec24B	R.DSRPLS*PILHIVK.D	1.23 ± 0.13	
IPI00032003	Emerin	R.TY*GEPESAGPSR.A	4.72 ± 0.60	
IPI00045486	Erbin	R.AQIPEGDY*LSYR.E	1.69 ± 0.16	8
IPI00045486	Erbin	K.DFNLPDY*DLNVEER.L	4.07 ± 0.63	
IPI00045486	Erbin	R.TY*SIDGPNASR.P	> 20	
IPI00045486	Erbin	N.Y*SQIHPPQASVAR.H	1.66 ± 0.12	
IPI00215637	DDX3	K.DKDAY*SSFGSR.S	0.74 ± 0.08	8
IPI00101968	Hypothetical protein	R.FQDVGPQAPVGSVY*QK.T	2.42 ± 0.19	
IPI00233255	SHP-2	R.VY*ENVGLMQQK.H	0.68 ± 0.20	42
IPI00297169	SLP-76	N.SNSMY*IDRPPSGK.T	0.66 ± 0.15	
IPI00329789	ZAP-70	K.ALGADDSY*Y*TAR.S	1.47 ± 0.27	43
IPI00329789	ZAP-70	R.IDTLNSDGY*TPEPAR-.I	1.22 ± 0.12	43
IPI00329789	ZAP-70	R.PMPMDTSVY*ESPY*SDPEELKDK-.K	1.68 ± 0.14	43,44
IPI00003406	Drebrin	R.SPS*DSSTASTPVAEQIER.A	1.74 ± 0.11	
IPI00012442	G3BP-1	K.SSS*PAPADIAQTVQEDLR-.T	0.77 ± 0.07	45
IPI00015029	Telomerase binding protein p23	K.DWEDDS*DEDMSNFDR.F	1.05 ± 0.06	46

^aPeriod indicates a tryptic cleavage site; dash indicates a C-terminal residue.

^bRatio of intensities of phosphopeptides after 10-min and 2-min treatments with pervanadate.

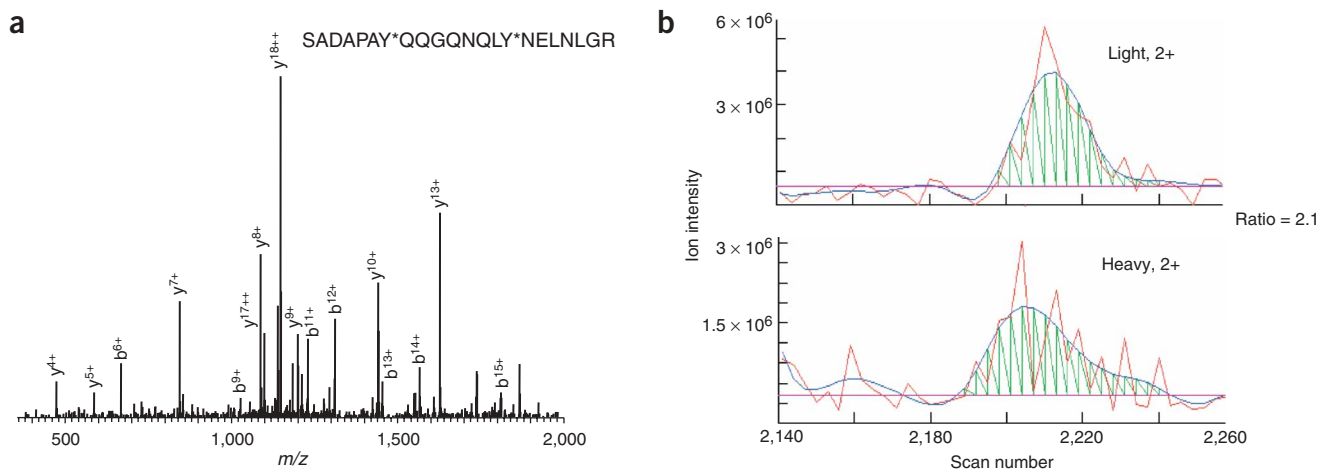


Figure 3 | Quantitative phosphopeptide analysis. **(a)** MS/MS of the CD3 ζ -driven peptide SADAPAY*QQGQNQLY*NELNLGR. Its characteristic peptide bond fragment ions, type b and type y ions, are labeled. **(b)** Reconstructed ion chromatogram of the precursor ion (m/z 778.3) and its heavy version (m/z 782.8) using ASAPRatio program. The program draws a smoothed chromatogram based on the ion signal and then calculates the ratio of the peak areas. Raw chromatograms are plotted in red, smoothed chromatograms in blue and areas used for calculating abundance ratio of the charge state in green.

open source software tools for peptide and protein validation (available from the Seattle Proteome Center website; <http://www.proteomecenter.org>). A peptide probability PeptideProphet score of 0.9 was chosen as the cutoff for tyrosine phosphopeptides. Associated with this cutoff, the predicted false-positive error rate is less than 1% (ref. 24). Serine/threonine phosphopeptides usually have poorer cross-correlation and probability scores, owing to the predominant loss of neutral molecules phosphoric acid (−98 Da) and water (−18 Da)¹². Therefore, a peptide probability score of 0.5 was chosen as the cutoff. These serine/threonine phosphopeptides with probability scores between 0.5 and 0.9 are as a result constrained with the dominant neutral loss of 98 Da. A partial list of 24 proteins is shown in **Table 1**. The entire list of phosphoproteins identified along with their phosphorylation sites is available (**Supplementary Table 1** online).

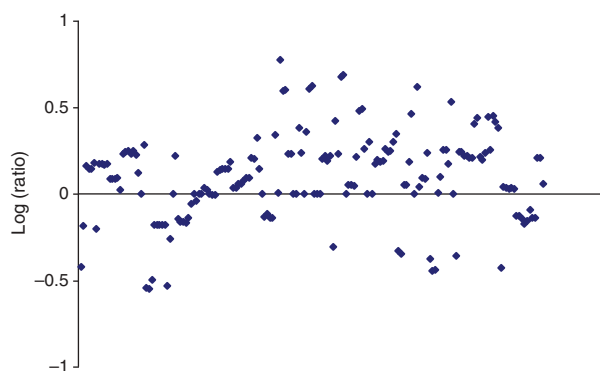


Figure 4 | Abundance ratios of tyrosine phosphopeptides isolated from T cells treated with pervanadate over 10 min or 2 min. Phosphopeptides with 10-min treatment were labeled with a light isotope (hydrogen, H), whereas those with 2 min treatment were labeled with a heavy isotope (deuterium, D). The ratios (light/heavy) are plotted as the logarithm scale. Repetitive measurements were made for most unique peptides. Log(ratio) values of above zero (majority) indicate phosphopeptides are more abundant with 10-min pervanadate treatment.

DISCUSSION

In initial experiments to develop the strategy, we attempted to covalently capture methylated phosphopeptides on an amino-functionalized solid-phase support through the same reaction but only achieved low yields. We determined that the EDC-activated reaction between phosphate and amino groups is kinetically extremely slow, and the density of amino groups on the solid phase is too low to carry out the reaction in a timely matter. Instead, the utility of soluble polyamines such as the dendrimer allows for homogeneous reaction with adequate amino groups in the solution. Large reagent excesses can still be used to drive reactions to completion. Noncovalently associated molecules and excess reagents can be removed by size selective methods such as size exclusion chromatography or a molecular weight cutoff filter device. The utility of functionalized synthetic soluble polymers like the dendrimer is not limited to the reaction reported here, but is applicable to any reaction in which a solid phase format cannot be achieved. Although the use of soluble polymers as support has been widely reported^{25,26}, this is the first report on the proteomic application. We expect broad applications using soluble polymers for sample processing in proteomics in the future, considering proteomics usually deals with low amounts of sample, and solid phase extraction approaches present a nontrivial problem owing to their heterogeneous nature. In this study, the dendrimer was chosen to capture the phosphopeptides owing to its discrete, controllable molecular architecture²⁷ and broad applications in combinatorial chemistry^{25,28}. It is conceivable, however, that other polyamines, such as polylysine and polyallylamine, may also be suitable for this application.

Our two-step approach of enriching tyrosine-phosphorylated proteins by immuno-affinity selection followed by chemical enrichment of phosphopeptides led to the MS detection of all known tyrosine phosphorylated residues in the (ITAMs)^{21,29} of the TCR's CD3 δ , CD3 ϵ , CD3 γ and CD3 ζ chains upon pervanadate treatment in a single experiment. For example, six tyrosine phosphorylation sites in the CD3 ζ chain identified in our experiment were discovered independently by different groups^{8,9,30}, including

one site (Y153), which was only very recently discovered⁹. The identified proteins also indicate that by using our strategy, a range of well and less well characterized signaling molecules are now detectable using an MS-based approach. These observations open new avenues of investigation, such as the MS-based study of signaling pathways upon cell perturbation with selective drugs.

The quantitative nature of the approach is apparent from the ability to obtain accurate measurements of small changes in the extent of phosphorylation. **Figure 3** illustrates the identification of a phosphopeptide and the quantification of its relative abundance in two cell states, in the case of a CD3 ζ -derived, doubly phosphorylated peptide, SADAPAY*QQGQNQLY*NELNLGR. MS/MS permits the unambiguous assignment of the peptide sequence and its tyrosine phosphorylation site. Quantitative information was obtained by reconstructing individual ion chromatograms of these two species and integrating the contour of the two respective peaks using the ASAPRatio program²⁰. In this case, the ratio (light:heavy) was 2.1, indicating that after a 10-min treatment with pervanadate, phosphorylation on two tyrosine residues of this peptide was increased twofold compared to the 2-min treatment.

Overall, tyrosine phosphorylation increased with increasing time of pervanadate treatment. This is indicated by the frequent observation of abundance ratios larger than 1.0 for phosphopeptides isolated after treatment for 10 min and 2 min, respectively (**Fig. 4**). For some phosphoproteins, the ratios observed for different peptides were not uniform, indicating differences in the kinetics of phosphorylation at specific tyrosine residues in the same protein (**Table 1**). Some phosphorylation sites appeared maximally induced within 2 min of stimulation (indicated by the ratio of phosphopeptides close to 1.0), whereas in the same protein, phosphorylation on other tyrosine residues further increased over the duration of the experiment. Only a few proteins had a decrease in tyrosine phosphorylation over extended pervanadate treatment (**Table 1**).

The present method can be used to detect concurrently serine/threonine/tyrosine phosphorylation sites in the same sample, which is illustrated by the observation that tyrosine-phosphorylated proteins are frequently also phosphorylated at serine and/or threonine residues (**Table 1** and **Supplementary Table 1**). Serine and threonine phosphorylation of signaling molecules and their binding partners is not as well understood as tyrosine phosphorylation, mainly because of a lack of suitable methods to detect and quantify these events. In contrast to tyrosine phosphorylation, for which extended treatment with pervanadate increased phosphorylation, many serine/threonine phosphorylation sites decreased over the duration of the treatment. For example, haematopoietic lineage cell-specific protein, HS1, a known tyrosine phosphoprotein, has recently been described to undergo serine/threonine phosphorylation as well³¹. We found, using our approach, one potential serine phosphorylation site whose extent of phosphorylation decreased when the cells were treated with pervanadate for 10 min. The biological significance of this and other similar observations remains to be determined.

In conclusion, the strategy presented here uses a simple chemical derivatization to selectively and efficiently enrich phosphopeptides and identify phosphorylation sites on a large scale. Owing to the nature of the chemical reaction, the approach applies generally to tyrosine/serine/threonine phosphorylation, and it is highly specific, that is, there was no detectable interference from other modifications such as glycosylation. Because the method relies on a

reversible covalent linkage, the phosphoramidate bond, it is well-suited for monitoring quantitative changes of *in vivo* phosphorylation events. In combination with an antiphosphotyrosine immunoaffinity step to enrich for tyrosine phosphorylated proteins the method allows the general and quantitative determination of the phosphorylation state of low-abundance PTK substrates in phosphotyrosine-regulated signaling networks.

METHODS

Isolation of tyrosine phosphoproteins. Immobilized 4G10 antiphosphotyrosine agarose beads and soluble 4G10 phosphotyrosine antibodies (Upstate Biotechnology, Inc.) were used for precipitation and immunoblot analysis of phosphotyrosine-containing proteins, respectively. Jurkat cell line, pervanadate/H₂O₂ stimulations, immunoprecipitation and western blot analysis have been described previously^{32,33}.

We grew $\sim 3 \times 10^8$ Jurkat cells in RPMI medium 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μ g/ml streptomycin sulfate, and 100 units/ml penicillin G in a 5% CO₂ incubator at 37 °C. Cells were stimulated with 50 μ M pervanadate solution, freshly prepared from sodium orthovanadate and H₂O₂ solutions, for the indicated times. We lysed the cells in 10 ml of lysis buffer containing 1% Triton, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.8). Subsequently, the postnuclear supernatant was subjected to antiphosphotyrosine immunoprecipitation with 2 ml of agarose-conjugated 4G10 monoclonal antibodies (for 1 ml of agarose beads, 1 mg of phosphotyrosine monoclonal antibody was covalently coupled on the beads) at 37 °C for 2 h. Settled beads were extensively washed with the lysis buffer, and bound proteins were eluted 5 times with 50 mM phenylphosphate, 200 mM NaCl. Proteins were concentrated with Microsep (10K) centrifugal filter device (Pall Filtron Co.).

Isotope tagging and enrichment of phosphopeptides. Protein mixtures were dissolved in 20 mM ammonium bicarbonate (pH 8.0) containing 0.1% RapiGest (Waters Co.), and heated at 95 °C for 5 min. Samples were reduced with 5 mM dithiothreitol for 30 min at 37 °C, and then alkylated with 15 mM iodoacetamide in the darkness (30 min, 15–25 °C). Proteins were digested with 1 μ g trypsin overnight at 37 °C. The resulting peptides were desalted using the MCX column (Waters Co.), lyophilized and reconstituted in 75 μ l of methanolic HCl, which was prepared by adding 100 μ l of acetyl chloride to 500 μ l of anhydrous methanol-d₀ or -d₄ (Cambridge Isotope Laboratories). The methyl esterification was allowed to proceed at 12 °C for 90 min. Solvent was removed in a SpeedVac concentrator (Savant), and peptide methyl esters were dissolved in 40 μ l of reaction solution containing 50 mM EDC, 100 mM imidazole, 200 mM MES (pH 6.0) and 9 mg of PAMAM dendrimer Generation 5 (final concentration of amine group is 1 M; dendrimer was supplied as a 10% (wt/vol) solution in methanol (Sigma-Aldrich) and methanol was removed *in vacuo* before use). The reaction was allowed to stand at room temperature with vigorous shaking for 10 h and the solution was transferred to a Biomax filter device (5-kDa cutoff; Millipore). Dendrimer-bound phosphopeptides were washed with 500 μ l of 3 M NaCl, 30% methanol and water several times, and the filtrates were discarded to remove nonspecifically bound nonphosphopeptides. Finally, 10% TFA was added, and the reactions were incubated at room temperature for 30 min to recover

phosphopeptides. The polymer was washed twice with 30% methanol and the filtrates were combined and dried for LC-MS analysis. For the Jurkat T cell sample, half of the final product was used for one mass spectrometric analysis.

LC-MS and database analysis. LCQ Deca XP quadrupole ion trap and LTQ quadrupole linear ion trap mass spectrometers (Thermo-electron) were used with a HP 1100 solvent delivery system (Agilent). Peptides were pressure-loaded on a capillary reverse-phase C₁₈ column (75- μ m inner diameter and 12 cm of bed length). Peptides were eluted from the capillary column at a flow rate of 200–300 nl/min to the mass spectrometer through an integrated electrospray emitter tip. Both MS and MS/MS spectra were acquired with the instrument operating in the data-dependent mode of one MS scan followed by two MS/MS scans. All MS/MS spectra were searched against International Protein Index (IPI) database using the SEQUEST algorithm³⁴. Static modifications were made on Asp, Glu, C-terminal (+14 and +17 for light and heavy isotopic labeling, respectively), and Cys residues (+57 for carboxyamino-methylation); Variable modifications of Ser, Thr and Tyr (+80) were permitted to allow for the detection of phosphorylation. A protein probability^{24,35} score of at least 0.5 was used to filter the data, followed by manual validation. Quantitative data analysis was performed using an in-house ASAPRatio program²⁰.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

This project has been funded in part with Federal funds from the National Heart, Lung and Blood Institute, National Institutes of Health, under contract No. N01-HV-28179. W.A.T. is a Damon Runyon Fellow supported by the Damon Runyon Cancer Research Foundation (DRG 1740-02).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 16 March; accepted 20 June 2005

Published online at <http://www.nature.com/naturemethods/>

- Pawson, T. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* **116**, 191–203 (2004).
- Jumaa, H., Hendriks, R.W. & Reth, M. B cell signaling and tumorigenesis. *Annu. Rev. Immunol.* **23**, 415–445 (2005).
- Kalume, D.E., Molina, H. & Pandey, A. Tackling the phosphoproteome: tools and strategies. *Curr. Opin. Chem. Biol.* **7**, 64–69 (2003).
- Loyet, K.M., Stults, J.T. & Arnott, D. Mass spectrometric contributions to the practice of phosphorylation site mapping through 2003: a literature review. *Mol. Cell Proteomics* **4**, 235–245 (2005).
- Zappacosta, F., Huddleston, M.J. & Annan, R.S. Comparative phosphorylation site mapping from gel-derived proteins using a multidimensional ES/MS-based approach. *Methods Mol. Biol.* **284**, 91–110 (2004).
- Mann, M. *et al.* Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol.* **20**, 261–268 (2002).
- Blagoev, B., Ong, S.E., Kratchmarova, I. & Mann, M. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat. Biotechnol.* **22**, 1139–1145 (2004).
- Brill, L.M. *et al.* Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T cells using immobilized metal affinity chromatography and tandem mass spectrometry. *Anal. Chem.* **76**, 2763–2772 (2004).
- Zheng, H., Hu, P., Quinn, D.F. & Wang, Y.K. Phosphotyrosine proteomic study of interferon signaling pathway using a combination of immunoprecipitation and immobilized metal affinity chromatography. *Mol. Cell. Proteomics* **4**, 721–730 (2005).
- Pandey, A. *et al.* Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. *Proc. Natl. Acad. Sci. USA* **97**, 179–184 (2000).
- Rush, J. *et al.* Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **23**, 94–101 (2005).
- Ficarro, S.B. *et al.* Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **20**, 301–305 (2002).
- Riggs, L., Seeley, E.H. & Regnier, F.E. Quantification of phosphoproteins with global internal standard technology. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **817**, 89–96 (2005).
- Haydon, C.E. *et al.* Identification of novel phosphorylation sites on *Xenopus laevis* Aurora A and analysis of phosphopeptide enrichment by immobilized metal-affinity chromatography. *Mol. Cell. Proteomics* **2**, 1055–1067 (2003).
- Zhou, H., Watts, J.D. & Aebersold, R. A systematic approach to the analysis of protein phosphorylation. *Nat. Biotechnol.* **19**, 375–378 (2001).
- Oda, Y., Nagasu, T. & Chait, B.T. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* **19**, 379–382 (2001).
- Goodlett, D.R. *et al.* Differential stable isotope labeling of peptides for quantitation and de novo sequence derivation. *Rapid Commun. Mass Spectrom.* **15**, 1214–1221 (2001).
- Chu, B., Wahl, G.M. & Orgel, L.E. Derivatization of unprotected polynucleotides. *Nucleic Acids Res.* **11**, 6513–6529 (1983).
- Gygi, S.P. *et al.* Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17**, 994–999 (1999).
- Li, X., Zhang, H., Ranish, J.A. & Aebersold, R. Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry. *Anal. Chem.* **75**, 6648–6657 (2003).
- Weiss, A. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell* **73**, 209–212 (1993).
- Alarcon, B., Gil, D., Delgado, P. & Schamel, W.W. Initiation of TCR signaling: regulation within CD3 dimers. *Immunol. Rev.* **191**, 38–46 (2003).
- Marie-Cardine, A. & Schraven, B. Coupling the TCR to downstream signalling pathways: the role of cytoplasmic and transmembrane adaptor proteins. *Cell. Signal.* **11**, 705–712 (1999).
- Nesvizhskii, A.I., Keller, A., Kolker, E. & Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75**, 4646–4658 (2003).
- Janda, K.D. & Han, H. Combinatorial chemistry: a liquid-phase approach. *Methods Enzymol.* **267**, 234–247 (1996).
- van Heerbeek, R., Kamer, P.C., van Leeuwen, P.W. & Reek, J.N. Dendrimers as support for recoverable catalysts and reagents. *Chem. Rev.* **102**, 3717–3756 (2002).
- Bosman, A.W., Janssen, H.M. & Meijer, E.W. About dendrimers: structure, physical properties and applications. *Chem. Rev.* **99**, 1665–1688 (1999).
- Kim, R.M. *et al.* Dendrimer-supported combinatorial chemistry. *Proc. Natl. Acad. Sci. USA* **93**, 10012–10017 (1996).
- Love, P.E. & Shores, E.W. ITAM multiplicity and thymocyte selection: how low can you go? *Immunity* **12**, 591–597 (2000).
- Kersh, E.N., Shaw, A.S. & Allen, P.M. Fidelity of T cell activation through multistep T-cell receptor zeta phosphorylation. *Science* **281**, 572–575 (1998).
- Ruzsanic, M., Penzo, D. & Pinna, L.A. Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem. J.* **364**, 41–47 (2002).
- Wienands, J., Larbolette, O. & Reth, M. Evidence for a preformed transducer complex organized by the B cell antigen receptor. *Proc. Natl. Acad. Sci. USA* **93**, 7865–7870 (1996).
- Baumann, G. *et al.* *In vitro* characterization of major ligands for Src homology 2 domains derived from protein tyrosine kinases, from the adaptor protein SHC and from GTPase-activating protein in Ramos B cells. *Eur. J. Immunol.* **24**, 1799–1807 (1994).
- Eng, J.K., McCormack, A.L. & Yate, J.R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976–989 (1994).
- Keller, A., Nesvizhskii, A.I., Kolker, E. & Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **74**, 5383–5392 (2002).
- Lin, Y.H. *et al.* Regulation of cell migration and survival by focal adhesion targeting of Lasp-1. *J. Cell Biol.* **165**, 421–432 (2004).
- Butch, E.R. & Guan, K.L. Characterization of ERK1 activation site mutants and the effect on recognition by MEK1 and MEK2. *J. Biol. Chem.* **271**, 4230–4235 (1996).
- Salomon, A.R. *et al.* Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc. Natl. Acad. Sci. USA* **100**, 443–448 (2003).
- Kashige, N., Carpino, N. & Kobayashi, R. Tyrosine phosphorylation of p62dok by p210bc-*abl* inhibits RasGAP activity. *Proc. Natl. Acad. Sci. USA* **97**, 2093–2098 (2000).
- de Aoz, I. *et al.* Tyrosine phosphorylation of the CD3- ϵ subunit of the T-cell antigen receptor mediates enhanced association with phosphatidylinositol 3-kinase in Jurkat T cells. *J. Biol. Chem.* **272**, 25310–25318 (1997).

41. Teng, J.M. *et al.* Phosphorylation of each of the distal three tyrosines of the CD28 cytoplasmic tail is required for CD28-induced T cell IL-2 secretion. *Tissue Antigens* **48**, 255–264 (1996).
42. MacGillivray, M. *et al.* The protein tyrosine phosphatase SHP-2 regulates interleukin-1-induced ERK activation in fibroblasts. *J. Biol. Chem.* **278**, 27190–27198 (2003).
43. Watts, J.D. *et al.* Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. *J. Biol. Chem.* **269**, 29520–29529 (1994).
44. Pelosi, M. *et al.* Tyrosine 319 in the interdomain B of ZAP-70 is a binding site for the Src homology 2 domain of Lck. *J. Biol. Chem.* **274**, 14229–14237 (1999).
45. Beausoleil, S.A. *et al.* Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. USA* **101**, 12130–12135 (2004).
46. Kobayashi, T. *et al.* Regulation of cytosolic prostaglandin E synthase by phosphorylation. *Biochem. J.* **381**, 59–69 (2004).