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Phosphospecific proteolysis for mapping sites of protein phosphorylation

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Protein phosphorylation is a dominant mechanism of information transfer in cells, and a major goal of current proteomic efforts is to generate a system-level map describing all the sites of protein phosphorylation. Recent efforts have focused on developing technologies for enriching and quantifying phosphopeptides. Identification of the sites of phosphorylation typically relies on tandem mass spectrometry to sequence individual peptides. Here we describe an approach for phosphopeptide mapping that makes it possible to interrogate a protein sequence directly with a protease that recognizes sites of phosphorylation. The key to this approach is the selective chemical transformation of phosphoserine and phosphothreonine residues into lysine analogs (aminoethylcysteine and β-methylaminoethylcysteine, respectively). Aminoethylcysteine-modified peptides are then cleaved with a lysine-specific protease to map sites of phosphorylation. A blocking step enables single-site cleavage, and adaptation of this reaction to the solid phase facilitates phosphopeptide enrichment and modification in one step.

Much of the complexity of higher organisms is believed to reside in the specific post-translational modification of proteins[1]. Protein phosphorylation is the most ubiquitous such modification; almost 2% of the human genome encodes protein kinases and an estimated one-third of all proteins are phosphorylated[2]. Owing to the importance of protein phosphorylation in regulating cellular signaling, there is intense interest in developing technologies for mapping phosphorylation events[3,4].

Existing approaches for phosphorylation site mapping rely largely on the use of tandem mass spectrometry (MS/MS) to sequence individual peptides. Despite the power of this approach, MS/MS of phosphopeptides remains challenging[5–10] because of (i) the signal suppression of phosphate-containing molecules in the commonly used positive detection mode, (ii) the inherent lability of the phosphate group upon collision induced dissociation (CID) and (iii) the difficulty of achieving full sequence coverage, especially for long peptides, peptides present in low abundance and peptides phosphorylated at substoichiometric levels—all of which are common for phosphopeptides. The challenge of mapping phosphorylation sites is highlighted by recent efforts to enrich phosphopeptides from complex mixtures. Although these strategies have provided powerful tools for purifying phosphopeptides, the next step—identifying the precise site of phosphorylation—often fails for many of the peptides that are recovered[7,8].

Currently, the first step in mapping the phosphorylation sites of a protein is to digest the phosphoprotein with a protease (*e.g.*, trypsin) that generates smaller peptide fragments for sequencing. We reasoned that this process would be more informative if a protease that specifically cleaves its substrates at the site of phosphorylation were used. Such a digestion would selectively hydrolyze the amide bond adjacent to each phosphorylated residue, facilitating identification of the phosphorylation site directly from the cleavage pattern without the sequencing of any individual peptide (*e.g.*, from an MS fingerprint specifying the exact masses of the cleavage products). Phosphospecific cleavage would also facilitate the interpretation of MS/MS spectra, because the C-terminal residue would always be the phosphorylated residue, resulting in a unique y_1 ion. In this regard, it is often possible to obtain tandem mass spectra of a phosphopeptide, but still fail to localize the phosphoamino acid within that sequence[7,8]. Unfortunately, no protease is known that selectively recognizes a phosphorylated amino acid, or any other post-translational modification.

To address this problem, we developed a strategy for specific proteolysis at sites of serine and threonine phosphorylation. This approach relies on the well-established β-elimination of phosphoserine residues to generate dehydroalanine under basic conditions (phosphothreonine is converted to β-methyldehydroalanine). Similar chemistry has been used to enrich and quantify phosphoproteins for traditional trypsin digestion and MS/MS sequencing[8,11–15]. In the next step, dehydroalanine acts as a Michael acceptor for cysteamine, generating an aminoethylcysteine (Aec) residue (for phosphothreonine, β-methylaminoethylcysteine is generated) (**Fig. 1a**). Because aminoethylcysteine is isosteric with lysine, proteases that recognize lysine (*e.g.*, trypsin, Lys-C and lysyl endopeptidase) will cleave proteins at this residue.

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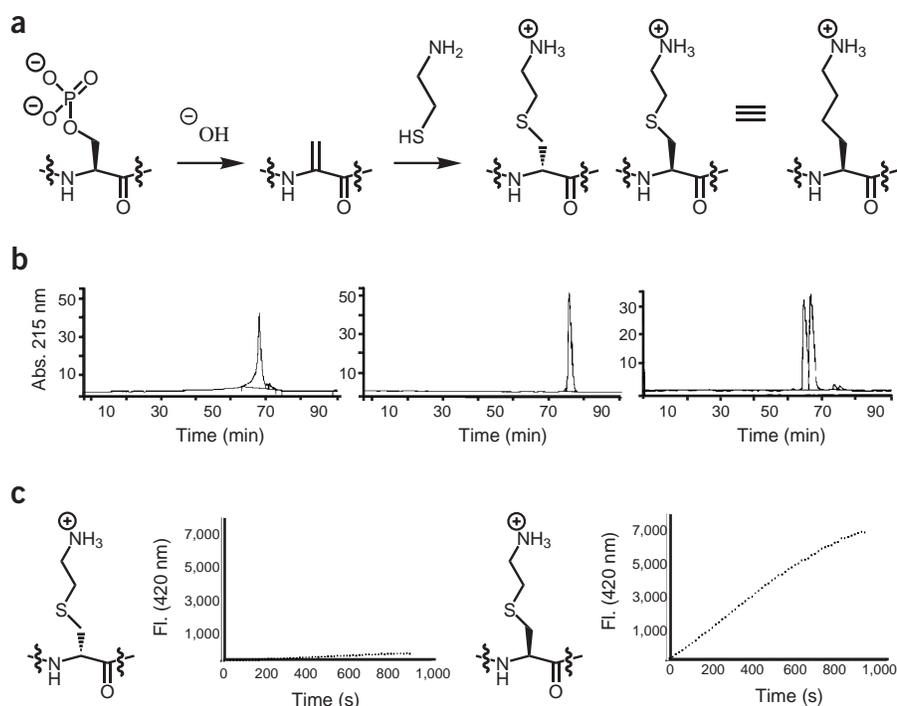
Figure 1 Aminoethylcysteine modification of phosphoserine. (**a**) Scheme for transformation of phosphoserine residues to dehydroalanine, then aminoethylcysteine. (**b**) HPLC traces of crude reactions cleanly converting phosphoserine peptide ZFRP**pS**GFY*D (left) to dehydroalanine (middle) then aminoethylcysteine (right). (**c**) Reaction progress curve for the hydrolysis of (S)-aminoethylcysteine (left) and (R)-aminoethylcysteine derivatives of peptide ZFRPpSGFY*D by trypsin as monitored by fluorescence resonance energy transfer (FRET).

RESULTS

Modification and digestion of model substrates

A panel of seven phosphoserine and two phosphothreonine peptides was chosen to demonstrate the feasibility of this approach. Extensive peptide degradation was found to result when standard β-elimination conditions (~1M hydroxide, 42–55 °C, >1 h) were applied[8,15,16]. Several parameters were critical for achieving nearly quantitative β-elimination without peptide hydrolysis, including reaction temperature, duration, solvent, basicity and order of addition[13,14] (see Methods). By this means, each peptide was cleanly converted into its aminoethylcysteine or β-methylaminoethylcysteine analog (**Fig. 1**, **Table 1** and **Supplementary Fig. 1** online). Digestion of the aminoethylcysteine modified peptides with Lys-C or trypsin liberated peptide fragments corresponding to selective cleavage at the site of serine phosphorylation (**Table 1**). Surprisingly, β-methylaminoethylcysteine was also found to be an efficient substrate for Lys-C and lysyl endopeptidase, generating peptide fragments corresponding to specific cleavage at the former site of threonine phosphorylation (**Table 1** and **Supplementary Fig. 2** online); trypsin cleaved at this residue less efficiently. In this way, site-specific modification combined with proteolytic digestion allows for the unambiguous identification

of serine and threonine phosphorylation sites from the exact masses of the liberated fragments.

To further explore the potential of this strategy for mapping phosphorylation sites, we selected two model proteins (α- and β-casein) that contain three and five sites of phosphorylation, respectively. Each protein was subjected to aminoethylcysteine modification followed by digestion with trypsin. One pmol of each digested protein was separated by nanoflow liquid chromatography (LC) on a nano-C18 column and then directly analyzed by online LC-MS, and MS/MS on a quadrupole orthogonal time-of-flight (TOF) spectrometer. Eight peptides were identified by mass fingerprinting (electrospray ionization (ESI)-MS) corresponding to direct cleavage at all eight predicted phosphorylation sites of the two proteins (**Table 2**). For example, for β-casein, phosphorylation has been described for serine residues in positions 15, 17, 18, 19 and 35. Accordingly, after aminoethylcysteine modification and digestion, peptides were observed containing C-terminal Aec residues corresponding to Arg1-Aec15, Arg1-Aec17, Arg1-Aec18 and Arg1-Aec19. The phosphorylation site at position 35 was assigned from the presence of the peptide Glu36-Lys48, whose N terminus can be attributed to cleavage at the adjacent Aec35 residue.

The identity of many of these aminoethylcysteine-containing peptides was confirmed by LC-MS/MS sequencing as listed in **Table 2**. Peptides containing aminoethylcysteine were found to produce typical peptide MS/MS fragmentation patterns that were readily interpretable. For example, the tandem mass spectrum for the peptide containing the putative phosphorylation site at position 15 in β-casein is shown in **Figure 2a**. Importantly, the characteristic y_1 ion at m/z 165.1 that results from a loss of a C-terminal aminoethylcysteine residue appears as a highly abundant product ion in this MS/MS spectrum and other CID spectra of peptides containing this C-terminal

Table 1 Results of aminoethylcysteine modification for model peptide substrates

| Sequence | Experimental Mass **M** (Calculated Mass **M**) | | |
	Dehydroalanine	Aminoethylcysteine	Lys-C Digest
GRTGRRN**pS**IHDIL	1,475.4 (1,476.6)	1,554.6 (1,553.8)	609.4 (609.7)
DLDVPIPGRFDRRV**pS**VAAE	2,093.0 (2,094.4)	2,170.6 (2,171.5)	1,800.0 (1,801.1)
SLRRS**pS**C*FGGRIDRIGAQSGLGC*NSFRY	3,140.4 (3,142.4)	3,218.2 (3,219.5)	2,471.8 (2,473.6)
KRP**pS**QRHGSKY-NH₂	1,324.4 (1,324.5)	1,402.2 (1,401.6)	711.6 (711.8)
LRRA**pS**LG	753.6 (753.9)	830.6 (831.0)	660.4 (660.8)
ZFRP**pS**GFY*D	1,133.7 (1,134.2)	1,210.7 (1,211.3)	684.6 (683.8)
ZFRP**pT**GFY*D	1,147.52 (1,147.47)	**1,224.54** (1,224.50)	**697.35** (697.34)
RRA**pS**PVA	737.43 (737.43)	**814.53** (814.46)	547.32 (547.22)
KR**pT**IRR	810.56 (810.53)	887.62 (887.56)	n/a

Molecular weights were determined by ESI-MS on a Waters Micromass ZQ instrument, and exact masses by LC-ESI-MS recorded on a QSTAR Pulsar i instrument or by MALDI-MS recorded on a Voyager DESTR plus instrument. All masses listed in bold letters were additionally confirmed by LC-MS/MS. Abbreviations: K*, aminoethylcysteine; M*, methionine sulphone; C*, cysteic acid; Y*, 3-nitrotyrosine; Z, 2-aminobenzoic acid.

Table 2 Results of aminoethylcysteine modification and proteolytic digestion for α-casein, β-casein, β-tubulin and GRK2

Protein	Residues	Peptide sequence	M_r Obs. (Calcd.)
α$_{s1}$-casein	43–58	DIG**K***E**K***TEDQAM*EDIK	**1,916.74** (1,916.78)
α$_{s1}$-casein	47–58	(**K***)E**K***TEDQAM*EDIK	**1,485.53** (1,485.59)
α$_{s1}$-casein	49–58	(**K***)TEDQAM*EDIK	**1,210.48** (1,210.50)
α$_{s1}$-casein	106–119	VPQLEIVPN**K***AEER	**1,638.84** (1,638.84)
α$_{s1}$-casein	106–115	VPQLEIVPN**K***	**1,153.58** (1,153.61)
α$_{s2}$-casein	153–164	TVDM*E**K***TEVFTK	1,476.60 (1,476.66)
α$_{s2}$-casein	159–164	(**K***)TEVFTK	**723.36** (723.38)
β-casein	1–25	RELEELNVPGEIVE**K***L**K***K***K***EESITR	**3,037.39** (3,037.47)
β-casein	1–19	RELEELNVPGEIVE**K***L**K***K***	**2,322.11** (2,322.12)
β-casein	1–18	RELEELNVPGEIVE**K***L**K***	2,176.03 (2,176.07)
β-casein	1–17	RELEELNVPGEIVE**K***L**K***	**2,029.92** (2,030.01)
β-casein	1–15	RELEELNVPGEIVE**K***	**1,770.78** (1,770.88)
β-casein	33–48	FQ**K***EEQQQTEDELQDK	**2,039.80** (2,039.87)
β-casein	36–48	(**K***)EEQQQTEDELQDK	1,618.65 (1,618.69)
GRK2	666–677	NKPR**K***PVVELSK	**1,411.78** (1,411.80)
GRK2	668–677	PR**K***PVVELSK	**1,169.62** (1,169.66)
GRK2	671–677	(**K***) PVVELSK	**771.40** (771.45)
β-tubulin	404–416	DEMEF**K$_T$***EAESNMN	**1,604.52** (1,604.58)
β-tubulin	404–416	DEM**EF**K$_T$***EAESNMN	**1,620.60** (1,620.57)
β-tubulin	404–409	DEMEF**K$_T$***	829.34 (829.30)
β-tubulin	404–409	DEM**EF**K$_T$***	845.38 (845.30)
β-tubulin	417–426	DLV**K***EYQQYQ	**1,330.51** (1,330.58)
β-tubulin	421–426	(**K***) EYQQYQ	857.32 (857.36)
β-tubulin	417–420	DLV**K***	491.26 (491.24)

Molecular weights were determined by LC-ESI-MS recorded on a QSTAR instrument. All masses listed in bold letters were additionally confirmed by LC-MS/MS. Abbreviations: **K***, aminoethylcysteine; **K$_T$***, β-methylaminoethylcysteine; M*, methionine sulphone; M**, methionine sulfoxide; C*, cysteic acid; M_r, relative molecular mass; Obs., observed; Calcd., calculated.

residue. Because the mass of this y$_1$-ion (165.07) is unique and does not overlap with other fragment ions resulting from naturally occurring amino acids, we suggest that this ion may also be used for precursor ion scanning to increase the sensitivity of phosphopeptide detection in complex peptide mixtures[9,17]. Unlike existing precursor ion scanning approaches, the detection of this y$_1$ ion is not only indicative of the presence of a phosphoserine-containing peptide, but also positively identifies its precise position in the sequence (that is, at the C terminus).

During aminoethylcysteine modification, epimerization occurs at C$_α$ of the formerly phosphorylated amino acid, generating diastereomeric aminoethylcysteine peptides (R, S) in an approximately 1:1 mixture (**Fig. 1b**). The peptides containing the R stereochemistry at C$_α$ are substrates for lysine-specific proteases, whereas those with the S stereochemistry are not (**Fig. 1c**). As a consequence, cleavage occurs at approximately 50% of the sites for any given phosphopeptide under complete proteolysis conditions. For a single tryptic peptide containing multiple phosphorylation sites, this partial digestion generates a ladder of peptides corresponding to successive single cleavage at each of the phosphorylation sites (**Table 2**). This effect is illustrated for β-casein tryptic peptide Arg1-Arg25, which contains four phosphorylation sites within a five amino acid sequence. In practice, this obligatory partial digestion is advantageous for phosphopeptide mapping because it provides staggered and redundant mass information for multiply phosphorylated peptides.

Although introducing new cleavage sites can provide more information about phosphorylation sites, it can also increase the complexity of the resulting mass spectra. Several experiments were performed to

investigate the trade-off between these effects. First, two equivalent samples of β-casein, one of which was aminoethylcysteine-modified and one of which was untreated, were subjected to trypsin digestion and analyzed by matrix-assisted laser desorption ionization (MALDI)-MS (**Fig. 2b** and **Supplementary Fig. 3** online). In the MALDI spectrum from the aminoethylcysteine-derivatized sample, four modified phosphopeptides are detected as prominent ions at m/z 1,771.9, 2,031.1, 2,041.0 and 3,038.4 (residues 1–15, 1–17, 33–48 and 1–25, respectively). Three aminoethylcysteine peptides detected by LC-MS are not observed by MALDI-MS, and we believe this reflects their lower susceptibility to trypsin cleavage (e.g., two of the three peptides contain X-EE motifs that are known to slow trypsin cleavage). In the MALDI-MS spectrum from the untreated control sample, tryptic peptides containing the phosphorylation sites are not detected (**Fig. 2b**, inset). Thus, in the absence of aminoethylcysteine modification, none of the phosphopeptides are observed by MALDI-MS under the standard conditions used for most proteomic work (positive-ion reflectron mode with α-cyano-4-hydroxycinnamic acid (HCCA) as the matrix), whereas with chemical modification, four of these peptides are prominent ions in the digest mixture. Otherwise, the spectra are very similar in both the distribution of ions and their relative abundance (**Supplementary Fig. 3** online). Although experimental conditions are known that can facilitate the detection of phosphopeptides by MALDI-MS[18], we believe that aminoethylcysteine modification selectively enhances the positive-ion mode mass spectrometric response of formerly phosphorylated peptides[9]. Indeed, dilution experiments with these samples indicated that aminoethylcysteine-modified peptides could be identified from as little as 25 fmol of an unseparated tryptic digest by MALDI-MS (**Supplementary Fig. 4** online).

Mapping GRK2 phosphorylation of tubulin

In contrast to model phosphoproteins such as caseins, many cellular phosphoproteins contain features that make their direct analysis by mass spectrometry more challenging. Such samples are often phosphorylated substoichiometrically, and the phosphoprotein itself may represent only one component of a more complex mixture[6]. In addition, unrelated post-translational modifications as well as sequence variations can contribute to sample complexity. To investigate the applicability of our approach to samples of greater complexity, we chose to analyze the phosphorylation of the cytoskeletal component tubulin by the G protein–coupled receptor kinase 2 (GRK2). Although microtubules have been implicated in playing a role in G protein–coupled receptor endocytosis[19], their post-translational modifications and consequent functional roles in this process have only begun to be elucidated. GRK2 has recently been shown to associate with tubulin in vitro, localize with tubulin in vivo and phosphorylate tubulin heterodimers in an agonist-dependent fashion[20–22].

Bovine tubulin copurifies as a mixture of 21 charge variants of the approximately 12 genetic sub-isoforms of the major α- and β-tubulin
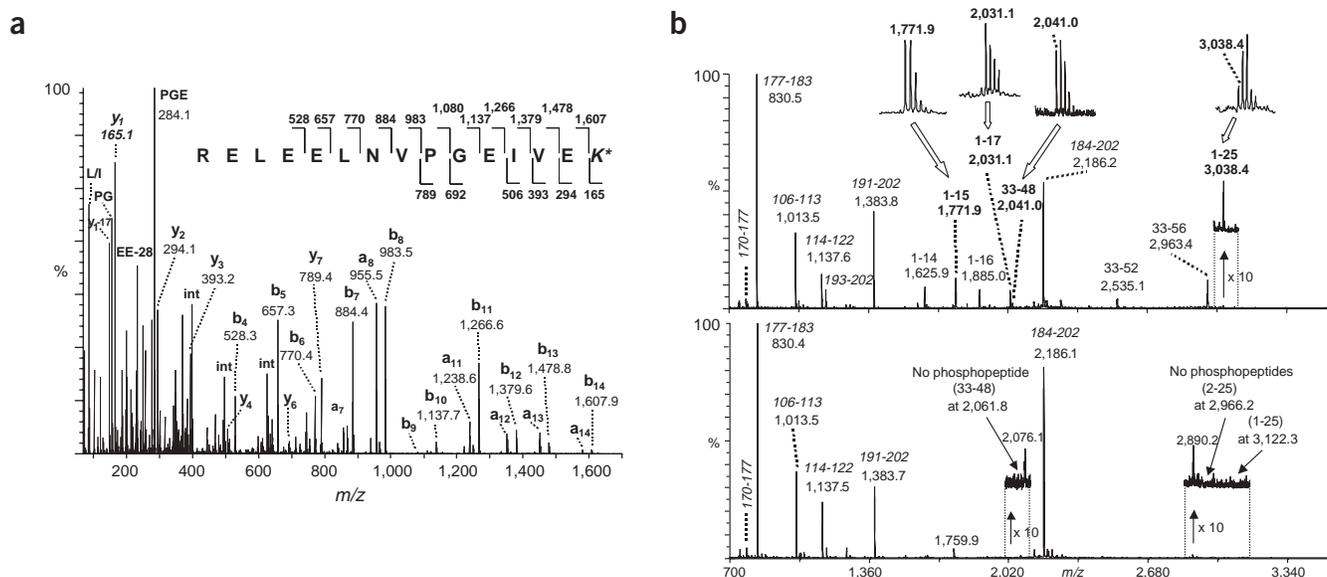
Figure 2 Mass spectra of aminoethylcysteine-modified β-casein. (**a**) ESI-MS/MS spectrum of peptide RELEELNVPGEIVEK* [residues Arg(1)-Lys*(15)] obtained after aminoethylcysteine modification of phosphoserine residues and digestion of β-casein with trypsin/Lys-C. The [M + 2H]$^{2+}$ at m/z 886.40^{2+} (M = 1770.79) was selected for CID. K* is aminoethylcysteine. (**b**) Top: β-casein (2 μg) was modified as aminoethylcysteine, digested with trypsin, and ~1 pmol analyzed by MALDI-MS. Masses in bold and magnified indicate aminoethylcysteine-modified peptides. Bottom: Unmodified β-casein (2 μg) was digested with trypsin, and ~1 pmol analyzed by MALDI-MS. Insets indicate that unmodified phosphoserine-containing peptides, predicted at m/z 2,061.8 (Phe33–Lys48) and at m/z 2,966.2 (Glu2–Arg25) or 3,122.3 (Arg1–Arg25) could not be detected in this spectrum.

isoforms[23,24]. Tubulin purified from bovine brain was phosphorylated by recombinant GRK2 *in vitro*. As the precise amino acid sequence of bovine tubulins is not known, we chose to look for phosphopeptides based on the known porcine sequences, reasoning that there would be few sequence differences between the two organisms. The mixture of phosphorylated and nonphosphorylated α- and β-tubulin isoforms in the presence of the GRK2 kinase was subjected to aminoethylcysteine modification, and then digested with different combinations of proteases to generate fragments suitable for mass spectrometry. We then searched the mass spectra for signature ions indicating aminoethylcysteine formation and cleavage after aminoethylcysteine sites. Digestion with Lys-C and Asp-N yielded two peptides with masses of 1,330.51 and 1,604.52, corresponding to peptides that span residues 417–426 and 404–416 of β-tubulin and that contain one aminoethylcysteine or one β-methylaminoethylcysteine residue, respectively (**Table 2**). Additional fragments corresponding to cleavage of these peptides directly at the two sites of phosphorylation were observed, and these fragment masses were sufficient to identify serine 420 and threonine 409 in β-tubulin as the phosphorylated residues. These assignments were confirmed by MS/MS sequencing of the derivatized peptides (**Supplementary Fig. 6** online), and none of these peptides were detected in control experiments lacking GRK2 treatment, confirming that GRK2 is the kinase responsible for this phosphorylation event. Interestingly, these phosphorylation sites occur near the C terminus of β-tubulin, within a highly acidic 6–7 kDa region that has been shown to undergo multiple post-translational modifications[23,24]. Lastly, we note that while this manuscript was in preparation, Yoshida *et al.* reported the identification of the analogous sites in porcine tubulin as targets of GRK2 (ref. 25). That study required a combination of radiolabeling, phosphoaminoacid thin-layer chromatography analysis, Edman degradation and the construction of chimeric substrates to pinpoint these sites.

As GRK2 itself is known to be a phosphoprotein[26], we looked for GRK2 phosphopeptides that might be present in our *in vitro* kinase reactions. After aminoethylcysteine modification and Lys-C digestion, we identified a peptide at m/z 1,412.78, which corresponds to a Lys-C fragment spanning residues 666–677 of GRK2 and contains one aminoethylcysteine residue (**Table 2**, **Fig. 3a** and **Supplementary Fig. 6** online). Additional fragments were observed corresponding to direct cleavage at the site of phosphorylation, identifying serine 670 as the site of modification. These results are consistent with earlier reports based on site-directed mutagenesis data that the S670A mutant is not phosphorylated in SF9 cells during protein expression[26].

In the kinase reactions used in this study, GRK2 is present at ~10% stoichiometry relative to the tubulin substrate. Moreover, inspection of the MALDI-MS spectrum of a GRK2 digest indicates that the phosphorylation of serine 670 itself is substoichiometric, as the aminoethylcysteine modified and nonphosphorylated (unmodified) peptides spanning residues 666–677 are equally abundant (**Fig. 3a**). To explore the sensitivity of our approach for identification of phosphorylation sites within a moderately complex and substoichiometric mixture, we carried out dilution experiments to determine the minimum amount of starting protein from a kinase reaction that can be carried through the aminoethylcysteine chemistry. Aminoethylcysteine modified peptides spanning GRK2 residues 666–677 and 668–677 were identified by LC-MS from 375 fmol of starting material; the peptide spanning residues 671–677 was detected at a level of 750 fmol. The tubulin phosphorylation sites could be identified at a level of 1.2 pmol of β-tubulin starting material. Lower starting amounts of tubulin were not tested, and the precise distribution of modified and cleaved peptides was dependent on the specific digest conditions used. However, this suggests that substoichiometric phosphorylation events can be analyzed via aminoethylcysteine modification in the absense of any pre-enrichment for phosphopeptides, although such enrichment would undoubtedly yield further
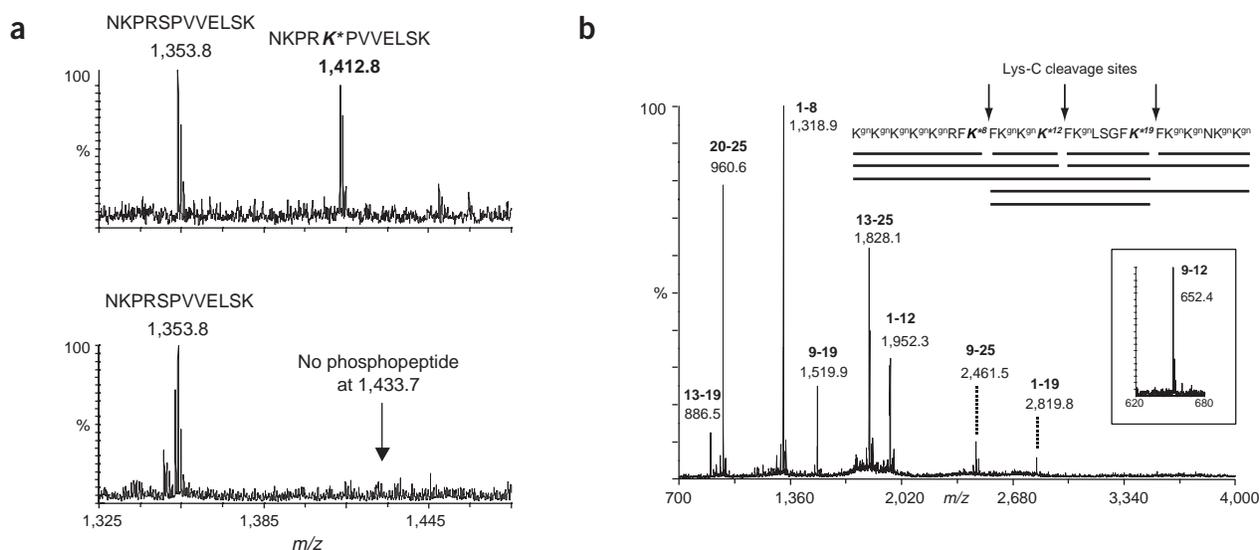
Figure 3 MALDI-MS spectra of aminoethylcysteine-modified phosphopeptides. (**a**) Top: The MALDI-MS spectrum displays molecular ions of peptides obtained after chemical modification of GRK2 and digestion with endoprotease Lys-C. The nonphosphorylated peptide observed at m/z 1,353.8 (residues 666–677) is in approximately equal abundance to the corresponding aminoethylcysteine modified peptide at m/z 1,412.8. Bottom: MALDI-MS of Lys-C digest of GRK-2 without chemical modification. The unmodified phosphopeptide (residues 666–677, containing phosphoserine 670) with an expected molecular ion at m/z 1,433.8 is not observed. (**b**) MALDI-MS spectrum after Lys-C cleavage of perguanidinated MARCKS substrate—a 25-residue peptide, $K^{gn}K^{gn}K^{gn}K^{gn}K^{gn}RFK^{*8}$ $FK^{gn}K^{gn}K^{*12}$ FK^{gn}LSGFK^{*19} FK^{gn}K^{gn}NK^{gn}K^{gn} (K^{gn}, homoarginine; K^*, aminoethylcysteine). An inset showing a mass range from m/z 620–680 reveals a cleavage product at m/z 652.4 (peptide Phe9–Lys*12) that was observed by MALDI-MS in a similar experiment after extended digestion with Lys-C.

improvements in sensitivity[6]. Lastly, similar titration experiments were performed with β-casein and the unfractionated digests were analyzed by MALDI-MS, yielding somewhat lower sensitivity (see **Supplementary Fig. 5** online).

Cleavage exclusively at phosphorylation sites

In some cases, it may be desirable to obtain cleavage exclusively at the phosphorylation site (not at lysine residues), generating larger fragments that might provide information about the gross topology of phosphorylation. The MARCKs substrate, a 25-residue peptide containing 12 lysine and 3 phosphoserine residues, was selected to explore the feasibility of achieving phosphoexclusive cleavage. To do this, we first converted lysine residues to homoarginine using *O*-methylisourea to block digestion at those sites with Lys-C. In addition to blocking proteolytic digestion, this modification has several practical advantages, including the enhancement of the ionization of homoarginine-containing peptides in MALDI[27–29] and reaction conditions that can facilitate the nearly quantitative (90–99%) guanidination of the lysine residues in full-length proteins[30,31].

After guanidination, the MARCKS substrate was modified as aminoethylcysteine, digested with Lys-C and subjected to mass analysis by MALDI (**Fig. 3b**). The MALDI mass spectrum from the digest exhibits eight prominent peaks corresponding to eight of nine possible combinations of cleavage at the three phosphorylation sites (**Supplementary Table 1** online). The smallest fragment, corresponding to cleavage at aminoethylcysteine residues 8 and 12 ($m/z = 652.4$), required longer digestion times and higher concentrations of protease to detect and is shown in the inset. No other major products were observed, confirming that homoarginine is not a substrate for Lys-C. Alternative chemistries are known for efficiently blocking lysine residues, and we have obtained similar results by acetylation (**Supplementary Table 1** and **Supplementary Fig. 7** online), although this modification may be less practical for full-length proteins.

A solid-phase aminoethylcysteine reaction

Although phosphorylation is among the most common post-translational modifications, phosphoproteins are often present at low abundance and phosphorylated substoichiometrically, making genome-wide phosphorylation analysis an analytical challenge. Several approaches have been proposed for the selective enrichment of phosphopeptides from complex mixtures[7,8,10], and the aminoethylcysteine derivatization described here should be compatible with the use of many of these strategies as a first step to enrich for phosphorylated peptides. However, we sought to investigate whether it would be possible to couple aminoethylcysteine modification directly to an approach for phosphopeptide enrichment. For this purpose, we adapted the aminoethylcysteine reaction to a solid phase, catch-and-release strategy to provide one-step modification and enrichment of phosphopeptides.

To prepare an appropriate solid-phase cysteamine equivalent, we loaded a polyethyleneglycol-polystyrene (PEG-PS) copolymer base resin with cystamine as the benzyl carbamate (**Fig. 4a**). This design incorporates two important features that facilitate aminoethylcysteine modification. First, the PEG-PS resin swells in both organic and aqueous solvents, allowing resin capture to be performed under conditions that have been optimized for the solution-phase chemistry. Secondly, the methoxybenzyl carbamate linkage is stable to the basic conditions of the β-elimination reaction, allowing for efficient peptide capture, but is highly acid-labile, facilitating aminoethylcysteine peptide release by brief treatment with trifluoroacetic acid (TFA).

We tested the ability of this reagent to selectively capture and modify phosphopeptides by incubating the resin with an approximately equimolar mixture of two nonphosphorylated peptides, one phosphotyrosine peptide and one phosphoserine peptide under β-elimination conditions for 1 h (**Fig. 4b**, upper). After incubation, high-performance liquid chromatography (HPLC) analysis of the flow-through indicated that the nonphosphorylated and phosphotyrosine peptides

remained intact, but the phosphoserine peak was absent, consistent with selective capture of the phosphoserine peptide (**Fig. 4b**, middle). Brief treatment with TFA released the phosphoserine peptide as the aminoethyl-cysteine modified diastereomer pair (**Fig. 4b**, lower), suitable for enzymatic phosphorylation mapping. This type of approach has the potential to allow proteolytic phosphorylation site mapping to be directly coupled to phosphopeptide enrichment from complex mixtures. The optimization and application of this solid-phase reaction is currently being explored.

DISCUSSION

We describe here an approach for mapping protein phosphorylation by direct enzymatic cleavage of polypeptides at the site of post-translational modification. To our knowledge, selective proteolysis at any site of protein post-translational modification has not been described previously, despite the large number of unique protease specificities found in nature. We believe that this type of approach will be a valuable complement to traditional MS/MS sequencing as a strategy

Figure 4 Solid-phase aminoethylcysteine reaction. (**a**) Scheme for the capture and modification of phosphoserine peptides using a solid-phase reagent. (**b**) Selective capture and modification of phosphoserine peptides using the cysteamine resin. Top, starting material; middle, flow-through; bottom, released aminoethylcysteine peptides. Peptide sequences (left to right, top): ZAAPpYGGY*D, ZAAPYGGY*D, ZFRPpSGFY*D, ZFRPFGFY*D. Z, 2-aminobenzoic acid; Y*, 3-nitrotyrosine.

for phosphorylation site mapping. In this regard, we have recently designed a protease that shows selectivity for phosphotyrosine substrates (Z.A.K. & K.M.S., unpublished observations). As O-glycosylated residues also undergo β-elimination under basic conditions[32], it may be possible to extend this strategy to map protein glycosylation in an analogous fashion. To achieve this, it will be important to investigate ways to distinguish between phosphorylation and glycosylation[32–34], such as pretreatment with an appropriate glycosidase or phosphatase. We envision a rapid expansion of enzyme tools for selective interrogation of the proteome, with tailor-made sequence specificity for all natural post-translational modifications.

METHODS

Materials. Sequencing grade trypsin, Lys-C and Asp-N were from Roche Diagnostics. Lysyl Endopeptidase was from Wako. Tentagel AC resin was from Advanced Chemtech. All peptides were from Anaspec or synthesized using standard Fmoc solid-phase chemistry. All other reagents were from Sigma unless otherwise noted and were of the highest grade commercially available.

Aminoethylcysteine modification of model peptides. The model phosphoserine peptides were dissolved in a 4:3:1 solution of H_2O/dimethyl sulfoxide (DMSO)/ethanol (50 μl). The β-elimination solution (saturated $Ba(OH)_2$ solution (23 μl) and 5 M NaOH (1 μl)) was added, and the reaction was incubated at room temperature. After 1 h, a 1 M solution of cysteamine in H_2O (50 μl) was added directly to this reaction and the reaction was incubated 3–6 h at room temperature. For peptides containing pSer-Pro sequences and pThr residues, the β-elimination reaction was allowed to proceed for 2 h at 37 °C. Reactions were analyzed by dilution into 1 ml H_2O/0.1% TFA and separation of the reaction products by reversed-phase HPLC on a Dynamax SD-200 solvent delivery system (Rainin) equipped with a Zorbax 300 C-18 9.4 mm × 25 cm column. Individual fractions were analyzed by ESI-MS offline using a Micromass ZQ (Waters) or by MALDI-MS and ESI-MS/MS (see below).

For site mapping, modified peptides were reconstituted in either 10 mM Tris, pH 8.5 (trypsin) or 10 mM Tris, pH 8.5, 1 mM EDTA (Lys-C) and digested overnight at 37 °C. Reactions were analyzed as above. For fluorescence resonance energy transfer (FRET) monitoring of the Lys-C digestion of
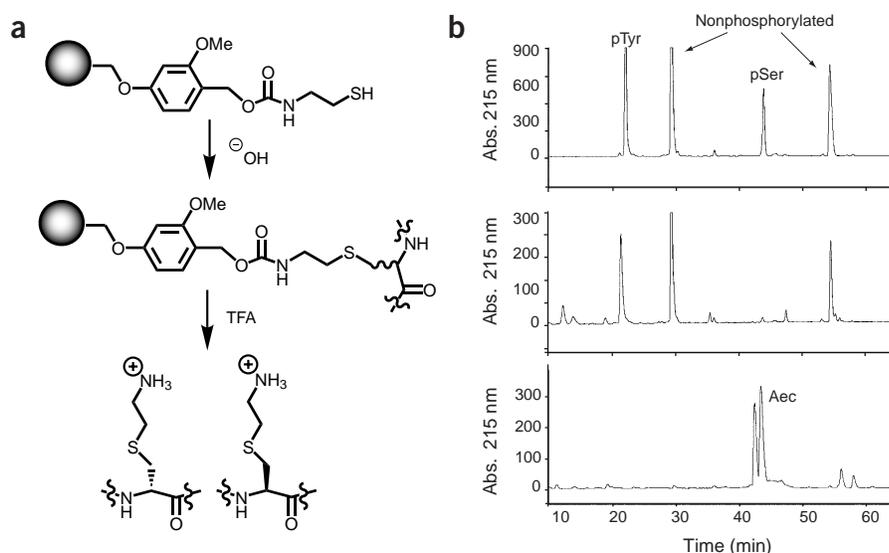
diastereomeric aminoethylcysteine peptides, peptide diastereomers (~5 μg) were separated by HPLC and digested with 5 μg trypsin. Reaction progress was monitored as emission at 420 nm after excitation at 320 nm in a SpectraMax GeminiXS fluorescence plate reader (Molecular Devices) as described[35].

Modification for phosphoexclusive cleavage. For guanidination reactions, the MARCKS substrate or β-casein was dissolved in 0.5 M O-methylisourea, pH 10.5 and incubated overnight at 37 °C essentially as described[28,29,36]. For acetylation reactions, the MARCKS substrate was dissolved in 100 mM $NaHCO_3$, pH 8.5 and treated with approximately 100 equivalents of sulfosuccinimidyl acetate (Pierce) for 2 h at room temperature to quantitatively acetylate lysine residues. Reactions were desalted by HPLC or dialysis and subjected to aminoethylcysteine modification and Lys-C digestion as above.

Mass spectra were obtained by MALDI-TOF MS on a Voyager DESTR plus (Applied Biosystems). All mass spectra were acquired in positive-ionization mode with reflectron optics. The instrument was equipped with a 337 nm nitrogen laser and operated under delayed extraction conditions in reflectron mode, a delay time of 190 ns and grid voltage 66–70% of full acceleration voltage (20–25 kV). For linear mode experiments, the delay time was 100 ns and the grid voltage 93.4% of the acceleration voltage. Before MALDI-MS analysis, the proteolytic reaction mixtures were desalted with reversed-phase Zip $Tips_{C18}$ (C-18 resin, Millipore). All peptide samples were prepared using a matrix solution consisting of 33 mM HCCA in acetonitrile/methanol (1:1; vol/vol); 1 μl of analyte (0.1–1 pmol of material) was mixed with 1 μl of matrix solution, and then air dried at room temperature on a stainless steel target. Typically, 50 laser shots were used to record each spectrum. The obtained mass spectra were externally calibrated with an equimolar mixture of angiotensin I, ACTH 1-17, ACTH 18-39 and ACTH 7-38.

Aminoethylcysteine modification of α- and β-casein. α-casein was pretreated with performic acid for 2 h to quantitatively oxidize cysteine residues[8]; β-casein, which contains no cysteine residues, was used as provided by the manufacturer. Proteins (~2 μg) were modified in 0.5 ml microcentrifuge tubes using the same conditions as described for peptides, except that the sample volume was adjusted to maintain a protein concentration ≥0.01 μg/μl. When the reaction was complete, reagents were removed by dialysis overnight against 1 liter of 20 mM Tris, pH 8.0 using 10,000 MWCO Slide-A-Lyzer Mini Dialysis units (Pierce). Other methods for reaction solvent exchange (e.g., gel filtration) led to

unacceptable sample losses. Dialyzed samples were transferred to a new 0.5 ml microcentrifuge tube, and the dialysis membrane was washed three times with 10 μl of 20 mM Tris, pH 8.0. Samples were concentrated to ~5 μl by Speed-Vac and 5 μl of acetonitrile was added as a denaturant. The sample mixture was heated to 65 °C for 10 min and then the digestion was initiated by the addition of 15 μl of 10 mM Tris, pH 8.0 containing trypsin or Lys-C at 1:10 enzyme to substrate by weight. Reactions were allowed to proceed approximately 6 h at 37 °C.

The proteolytic peptide mixtures (~1 pmol) were analyzed by MALDI-MS as described or by reversed-phase HPLC-MS/MS. Briefly, peptides were separated on an Ultimate nanocapillary HPLC system equipped with a PepMap C18 nano-column (75 μm internal diameter × 15 cm) (Dionex) and CapTrap Micro guard column (0.5 μl bed volume, Michrom). Peptide mixtures were loaded onto the guard column and washed with the loading solvent (H_2O/0.05 % formic acid, flow rate: 20 μl/min) for 5 min to remove salts and denaturing reagents, then transferred onto the analytical C18-nanocapillary HPLC column and eluted at a flow rate of 300 nl/min using the following gradient: 2% B (from 0–5 min), and 2–70% B (from 5–55 min). Solvent A consisted of 0.05% formic acid in 98% H_2O/2% acetonitrile and solvent B consisted of 0.05% formic acid in 98% acetonitrile/2% H_2O. The column eluant was directly coupled to the QSTAR quadrupole orthogonal TOF mass spectrometer (MDS Sciex) equipped with a Protana nanospray ion source. The nanospray needle voltage was typically 2300 V in the HPLC-MS mode. Mass spectra (ESI-MS) and tandem mass spectra (ESI-MS/MS) were recorded in positive-ion mode with a resolution of 12,000–15,000 full-width half-maximum (FWHM). For collision induced dissociation tandem mass spectrometry (CID-MS/MS), the mass window for precursor ion selection of the quadrupole mass analyzer was set to ±1 mass unit. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. The LC-MS runs on the QSTAR instrument were acquired in 'Information Dependent Acquisition' mode (advanced IDA), which allows the user to acquire MS/MS spectra based on an inclusion mass list and dynamic assessment of relative ion intensity. Spectra were calibrated in static nanospray mode using MS/MS fragment-ions of a renin peptide standard (histidine immonium-ion with m/z at 110.0713, and b_8-ion with m/z at 1,028.5312) providing a mass accuracy of ≤50 ppm.

Phosphorylation mapping of GRK2 and tubulin. N-terminal His6-tagged GRK2 was expressed in SF9 insect cells and purified using Ni-NTA beads (Qiagen) as described[37]. Bovine tubulin was a gift from Ron Vale. Tubulin (5 μM) and GRK2 (0.6 μM) were incubated in 100 μl of 20 mM HEPES, pH 7.4, 2.0 mM EDTA, 10 mM $MgCl_2$ containing 1 mM ATP. Kinase reactions were performed at 25 °C for 3 h[22], after which the reactions were desalted by microdialysis, subjected to aminoethylcysteine modification, digested with either Lys-C/trypsin or Lys-C/Asp-N and finally analyzed by LC-MS/MS and MALDI-MS as described above. In a similar fashion, purified GRK2 (~5 μg) was subjected to aminoethylcysteine modification, digested with Lys-C and then analyzed by mass spectrometry as described.

Resin synthesis. Resin was loaded using a modification of the procedure of Dorff[38]. Briefly, Tentagel AC resin (5 g) was swollen in anhydrous THF (75 ml) at room temperature under an inert atmosphere. 1,1 carbonyldiimidazole (2.5 g) was added and stirred for 3 h. The resin was filtered, washed with THF, diethyl ether and dried *in vacuo* overnight.

Before use, cystamine HCl salt (5 g) was dissolved in H_2O (45 ml), the pH was adjusted to 12 with NaOH and the cystamine was extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered, and the solvent removed *in vacuo* to give a clear oil. This oil (~1 g) was added to activated resin (2 g) swollen in THF (25 ml). *N*-methylmorpholine (2 ml) was added and the resin was heated to 60 °C for 4–6 h under an inert atmosphere.

The resin was filtered, washed with THF and diethyl ether, dried *in vacuo* and stored at –20 °C. Immediately before use, the resin was deprotected by brief treatment (15 min) with 100 mM dithiothreitol in H_2O to expose the cysteamine thiol. Quantification of resin loading with Ellman's reagent typically demonstrated 60–80% loading (0.20 to 0.25 mmol/g).

Solid-phase capture and modification of phosphoserine peptides. After deprotection, the resin was washed five times with H_2O and five times with 4:3:1

H_2O/DMSO/ethanol. Peptides were dissolved in 4:3:1 H_2O/DMSO/ethanol (250 μl) and added to 80 mg of resin swollen in the same. Saturated $Ba(OH)_2$ (225 μl) and 5 M NaOH (10 μl) were added and the reaction was incubated for 1 h at room temperature. The resin was then rinsed successively with H_2O, dimethylformamide, CH_2Cl_2 and diethyl ether, and dried overnight *in vacuo*. To release the peptides, the dried resin was suspended in 95:2.5:2.5 TFA/dimethylsulfide/H_2O (1 ml) for 15 min at room temperature. The resin was then filtered, washed three times with TFA (1 ml), and the filtrate was concentrated *in vacuo*. The released peptides were taken up in H_2O/0.1% TFA and analyzed by HPLC and MS as described.

Aminoethylcysteine modification protocol. Several parameters were found to be critical for successful aminoethylcysteine modification: (i) limiting hydroxide concentration to ~150 mM, (ii) adding barium as a specific catalyst for phosphate elimination[13], (iii) carrying out reactions in a previously optimized mixture of DMSO, water and ethanol[14], (iv) carefully limiting the reaction length and temperature (1 h at room temperature was sufficient for most phosphoserine peptides; 2 h at 37 °C was required for peptides containing phosphothreonine and phosphoserine residues N-terminal to proline), (v) performing the β-elimination and Michael addition steps consecutively, such that the addition of cysteamine to the basic reaction mixture in the second step reduces the pH of the reaction to ~8, and (vi) allowing the Michael addition step to proceed for up to 6 h for full modification of phosphothreonine peptides.

Protein samples for aminoethylcysteine modification are first desalted overnight by microdialysis against 2 liters water using 10,000 MWCO Slide-A-Lyzer Mini Dialysis units. Before use, dialysis units are rinsed extensively to remove any polymeric material that remains from the manufacturing process. After dialysis, dialyzed samples are transferred to 0.5 ml Eppendorf tubes, and the dialysis membrane is washed three times with 20 μl water. The combined dialysate is then concentrated by Speed-Vac to reduce volume to ~5 μl, with care not to concentrate to dryness. 5 μl of a 3:1 mixture of DMSO/ethanol is added directly to this sample. β-elimination is initiated by the addition of 4.6 μl saturated $Ba(OH)_2$ and 1 μl 500 mM NaOH. For most proteins, a 2-h incubation in a 37 °C water bath is recommended. At this stage, solutions of some full-length proteins may appear somewhat heterogeneous; this has no effect on the efficiency of the reaction, but gentle vortexing every 20 or 30 min is recommended to prevent excessive aggregation. After 2 h, the sample is placed at room temperature. While the sample is cooling (5–10 min), a 1 M solution of cysteamine HCl is freshly prepared, and 10 μl of this solution is added directly to the β-elimination reaction. This reaction is allowed to proceed 3–6 h at room temperature.

When the Michael addition reaction is complete, the protein solution is transferred to a rinsed mini-dialysis unit and dialyzed overnight against 2 L of 20 mM Tris, pH 8.0. The Eppendorf tube from the β-elimination reaction is rinsed three times with 15 ml of 20 mM Tris, pH 8.0 to ensure complete protein transfer. After dialysis, the protein is transferred to a new 0.5-ml Eppendorf tube, with careful rinsing of the dialysis membrane. This protein solution is then concentrated by Speed-Vac. This concentrated protein sample is then ready for digestion with appropriate proteases (*e.g.*, trypsin or Lys-C) and analysis by LC-MS/MS or MALDI-MS. In general, we find that Lys-C cleaves at modified sites somewhat more efficiently than trypsin, and that it is advisable to use slightly higher concentrations of protease than would be recommended for an ordinary trypsin digestion, although optimal digestion conditions vary substantially between samples.

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

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

1. Venter, J.C. *et al.* The sequence of the human genome. *Science* **291**, 1304–1351 (2001).
2. Manning, G., Whyte, D.B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298**, 1912–1934 (2002).
3. Shah, K. & Shokat, K.M. A chemical genetic screen for direct v-Src substrates reveals ordered assembly of a retrograde signaling pathway. *Chem. Biol.* **9**, 35–47 (2002).
4. Bishop, A.C. *et al.* A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* **407**, 395–401 (2000).
5. McLachlin, D.T. & Chait, B.T. Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol.* **5**, 591–602 (2001).
6. Mann, M. *et al.* Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol.* **20**, 261–268 (2002).
7. Zhou, H., Watts, J.D. & Aebersold, R. A systematic approach to the analysis of protein phosphorylation. *Nat. Biotechnol.* **19**, 375–378 (2001).
8. 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).
9. Steen, H. & Mann, M. A new derivatization strategy for the analysis of phosphopeptides by precursor ion scanning in positive ion mode. *J. Am. Soc. Mass Spectrom.* **13**, 996–1003 (2002).
10. Ficarro, S.B. *et al.* Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat. Biotechnol.* **20**, 301–305 (2002).
11. Meyer, H.E., Hoffmann-Posorske, E., Korte, H. & Heilmeyer, L.M. Jr. Sequence analysis of phosphoserine-containing peptides. Modification for picomolar sensitivity. *FEBS Lett.* **204**, 61–66 (1986).
12. Simpson, D.L., Hranisavljevic, J. & Davidson, E.A. Elimination and sulfite addition as a means of localization and identification of substituted seryl and threonyl residues in proteins and proteoglycans. *Biochemistry* **11**, 1849–1856 (1972).
13. Byford, M.F. Rapid and selective modification of phosphoserine residues catalysed by Ba2+ ions for their detection during peptide microsequencing. *Biochem. J.* **280 (Pt 1)**, 261–265 (1991).
14. Adamczyk, M., Gebler, J.C. & Wu, J. Selective analysis of phosphopeptides within a protein mixture by chemical modification, reversible biotinylation and mass spectrometry. *Rapid Commun. Mass Spectrom.* **15**, 1481–1488 (2001).
15. Goshe, M.B. *et al.* Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal. Chem.* **73**, 2578–2586 (2001).
16. Jaffe, H., Veeranna & Pant, H.C. Characterization of serine and threonine phosphorylation sites in beta-elimination/ethanethiol addition-modified proteins by electrospray tandem mass spectrometry and database searching. *Biochemistry* **37**, 16211–16224 (1998).
17. Annan, R.S., Huddleston, M.J., Verma, R., Deshaies, R.J. & Carr, S.A. A multidimensional electrospray MS-based approach to phosphopeptide mapping. *Anal. Chem.* **73**, 393–404 (2001).
18. Janek, K., Wenschuh, H., Bienert, M. & Krause, E. Phosphopeptide analysis by positive and negative ion matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **15**, 1593–1599 (2001).
19. Limas, C.J. & Limas, C. Involvement of microtubules in the isoproterenol-induced 'down'-regulation of myocardial beta-adrenergic receptors. *Biochim. Biophys. Acta* **735**, 181–184 (1983).
20. Haga, K., Ogawa, H., Haga, T. & Murofushi, H. GTP-binding-protein-coupled receptor kinase 2 (GRK2) binds and phosphorylates tubulin. *Eur. J. Biochem.* **255**, 363–368 (1998).
21. Pitcher, J.A. *et al.* The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J. Biol. Chem.* **273**, 12316–12324 (1998).
22. Carman, C.V., Som, T., Kim, C.M. & Benovic, J.L. Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. *J. Biol. Chem.* **273**, 20308–20316 (1998).
23. Banerjee, A. Coordination of posttranslational modifications of bovine brain alpha-tubulin. Polyglycylation of delta2 tubulin. *J. Biol. Chem.* **277**, 46140–46144 (2002).
24. Alexander, J.E. *et al.* Characterization of posttranslational modifications in neuron-specific class III beta-tubulin by mass spectrometry. *Proc. Natl. Acad. Sci. USA* **88**, 4685–4689 (1991).
25. Yoshida, N., Haga, K. & Haga, T. Identification of sites of phosphorylation by G-protein-coupled receptor kinase 2 in beta-tubulin. *Eur. J. Biochem.* **270**, 1154–1163 (2003).
26. Pitcher, J.A. *et al.* Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. *J. Biol. Chem.* **274**, 34531–34534 (1999).
27. Beardsley, R.L., Karty, J.A. & Reilly, J.P. Enhancing the intensities of lysine-terminated tryptic peptide ions in matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **14**, 2147–2153 (2000).
28. Beardsley, R.L. & Reilly, J.P. Optimization of guanidination procedures for MALDI mass mapping. *Anal. Chem.* **74**, 1884–1890 (2002).
29. Brancia, F.L., Oliver, S.G. & Gaskell, S.J. Improved matrix-assisted laser desorption/ionization mass spectrometric analysis of tryptic hydrolysates of proteins following guanidination of lysine-containing peptides. *Rapid Commun. Mass Spectrom.* **14**, 2070–2073 (2000).
30. Cupo, P., El-Deiry, W., Whitney, P.L. & Awad, W.M. Jr. Stabilization of proteins by guanidination. *J. Biol. Chem.* **255**, 10828–10833 (1980).
31. Kimmel, J.R. Guanidination of Proteins. *Methods Enzymol.* **11**, 584–589 (1967).
32. Mega, T., Nakamura, N. & Ikenaka, T. Modifications of substituted seryl and threonyl residues in phosphopeptides and a polysialoglycoprotein by beta-elimination and nucleophile additions. *J. Biochem. (Tokyo)* **107**, 68–72 (1990).
33. Wells, L.V. *et al.* Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. *Molecular and Cellular Proteomics* **1**, 791–804 (2002).
34. Greis, K.D. *et al.* Selective detection and site-analysis of O-GlcNAc-modified glycopeptides by beta-elimination and tandem electrospray mass spectrometry. *Anal. Biochem.* **234**, 38–49 (1996).
35. Meldal, M. & Breddam, K. Anthranilamide and nitrotyrosine as a donor-acceptor pair in internally quenched fluorescent substrates for endopeptidases: multicolumn peptide synthesis of enzyme substrates for subtilisin Carlsberg and pepsin. *Anal. Biochem.* **195**, 141–147 (1991).
36. Bonetto, V., Bergman, A.C., Jornvall, H. & Sillard, R. C-terminal sequence analysis of peptides and proteins using carboxypeptidases and mass spectrometry after derivatization of Lys and Cys residues. *Anal. Chem.* **69**, 1315–1319 (1997).
37. Kim, C.M., Dion, S.B., Onorato, J.J. & Benovic, J.L. Expression and characterization of two beta-adrenergic receptor kinase isoforms using the baculovirus expression system. *Receptor* **3**, 39–55 (1993).
38. Dorff, P.a.H. & Hauske, J.R. A Solid Phase CBZ Chloride Equivalent - A New Matrix Specific Linker. *Tetrahedron Lett.* **36**, 1589–1592 (1995).