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A semisynthetic epitope for kinase substrates

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Abstract

The ubiquitous nature of protein phosphorylation makes it challenging to map kinase-substrate relationships, which is a necessary step toward defining signaling network architecture. To trace the activity of individual kinases, we developed a semisynthetic reaction scheme, which results in the affinity tagging of substrates of the kinase in question. First, a kinase, engineered to use a bio-orthogonal ATPγS analog, catalyzes thiophosphorylation of its direct substrates. Second, alkylation of thiophosphorylated serine, threonine or tyrosine residues creates an epitope for thiophosphate ester-specific antibodies. We demonstrated the generality of semisynthetic epitope construction with 13 diverse kinases: JNK1, p38α MAPK, Erk1, Erk2, Akt1, PKCδ, PKCε, Cdk1/cyclinB, CK1, Cdc5, GSK3β, Src and Abl. Application of this approach, in cells isolated from a mouse that expressed endogenous levels of an analog-specific (AS) kinase (Erk2), allowed purification of a direct Erk2 substrate.

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AUTHOR CONTRIBUTIONS J.J.A. and K.M.S. wrote the manuscript. J.J.A. performed all experiments except those mentioned below. M.L. performed Erk2 substrate labeling reactions, C.S.B. performed mass spectrometry of modified peptides, J.L.P. expressed Cdc5, D.W. provided technical assistance for PKCδ experiments, A.H. constructed JNK1 plasmids, C.D.K. constructed the AS Erk2 mouse, W.C. constructed PKCδ plasmids, A.L.B. advised C.S.B., R.O.M. advised D.W. and W.C., and assisted in manuscript revision, R.J.D. advised A.H., S.M.H. advised M.L. and C.D.K. and K.M.S. advised J.J.A.

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Kinase-substrate interactions transduce extracellular signals into appropriate intracellular responses, and mapping these relationships is fundamental to understanding how signaling-network connectivity results in distinct biological outcomes. Yet owing to a paucity of techniques that permit association of an individual kinase with its direct substrates, a great many connections remain to be defined^{1,2}. Shared enzymology among protein kinase family members makes it difficult to follow the activity of a single kinase in the presence of all other cellular kinases. Protein chips³ circumvent this challenge by isolating a kinase and potential substrates from cellular complexity. But cellular components that impede substrate identification can also impose specificity, as kinase fidelity is often enforced through scaffolds⁴, cofactors and priming of nearby residues by phosphorylation⁵. Our goal is to develop bio-orthogonal chemical reactions, unique from the natural repertoire of cellular enzymology, to allow individual kinase substrates to be traced in the presence of signaling components that contribute to physiological specificity.

Specific kinase substrate labeling is achieved by engineering the kinase of interest to accept bio-orthogonal ATP analogs that are not used by the remainder of the kinome⁶. For example, AS kinases use bulky [γ -³²P]ATP analogs⁷ to produce radiolabeled substrates of a single kinase. Application of this strategy to yeast glutathione *S*-transferase–tagged or tandem affinity purification (TAP)-tagged libraries has yielded hundreds of Cdc28 (ref. ⁸) and Pho85 (ref. ⁹) substrate pairs. In these screens AS kinases had been added to yeast extracts containing candidate substrates, which can be purified by virtue of their genetically encoded affinity handles. But such libraries are not available for mammalian proteins and, without the aid of an affinity handle, identification of mammalian AS kinase substrates faces the same challenges as other phosphoproteomic studies, including the dynamic range of protein abundance and stoichiometric substrate phosphorylation¹⁰. In studies of mammalian AS kinase substrates this challenge has typically been addressed by immunoprecipitating the kinase followed by identification of bound substrates. For example, AS Erk2 substrates can be identified by performing kinase assays on immune complexes¹¹, which provides a necessary pre-enrichment of Erk2 binding proteins. Many kinase-substrate interactions are of low affinity, however, and are not amenable to this approach. Further, in all previous studies of AS kinase substrates, the kinase was either overexpressed or purified and added to cell extracts. Here we report two key advances toward the goal of unbiased identification of endogenous kinase substrates: substrate labeling in cells derived from a ‘gene knock-in’ AS kinase mouse¹²⁻¹⁴ and development of a method for affinity purification of AS kinase substrates.

To convert the phosphate, delivered specifically by an AS kinase into an affinity handle, we developed a semisynthetic reaction scheme. First, an AS kinase uses an N6-alkylated ATP γ S (A*TP γ S) to thiophosphorylate its substrates. Because all other kinases will instead transfer phosphate to their substrates, substituting a reactive sulfur in place of oxygen provides a unique starting point from which to differentiate AS kinase substrates from all other phosphoproteins. Reactions that can functionalize thiophosphate-containing proteins, facilitating their purification, will also modify other cellular nucleophiles. For example, alkylation of thiophosphate with the electrophilic tag *p*-nitrobenzylmesylate (PNBM) is concomitant with formation of undesired alkylation products resulting from derivatization of cysteine thiols. To specifically detect and purify thiophosphate reaction products, we developed polyclonal and monoclonal antibodies that discriminate thiophosphate esters from cysteine alkylation products (thioethers).

In our first report¹⁵, we had used this approach to detect substrates of the serine-threonine kinase, Cdk1. To further develop this strategy and expand the portion of the kinome tractable with this approach, we analyzed each of the reaction steps (Fig. 1a) for specificity and applicability to many kinase-substrate pairs. We screened diverse kinases for the ability to thiophosphorylate their protein substrates and found that the vast majority of kinases used

ATP γ S as a phosphodonor. We synthesized orthogonal A*TP γ S analogs and found them to be preferred substrates for AS kinases, permitting delivery of thiophosphate to individual kinase substrates. The resulting labeled kinase substrates, which contained modified serine, threonine and tyrosine residues in the context of diverse kinase consensus motifs, were all recognized by new thiophosphate ester-specific antibodies. Our initial hapten design presented the thiophosphate ester modification on a threonine backbone¹⁵; the elicited hapten-specific antibodies, however, suffered from either low yield of specific antibody or low affinity, and were not expected to recognize modified tyrosine residues. Here we designed a new hapten (Fig. 1b) containing only the minimal desired epitope in an effort to focus the immune response on the thiophosphate ester moiety. The elicited rabbit polyclonal and monoclonal antibodies are thiophosphate ester-specific, context-independent and capable of immunoprecipitation. Application of this semisynthetic immunoaffinity approach allowed us to purify the direct substrates of an AS kinase (Erk2), which was expressed at endogenous levels.

RESULTS

Generation of thiophosphate ester-specific IgG

We elicited rabbit α -hapten-immunoglobulin gamma (IgG) with a hapten conjugate, consisting of a *p*-nitrobenzylthiophosphate ester with a three-carbon linker to keyhole limpet hemocyanin (KLH; Fig. 1b). Affinity-purified polyclonal antibodies only recognized c-Jun-GST containing both thiophosphorylation and PNBM modifications (Fig. 1c), verifying that the antibodies discriminate thiophosphate alkylation products from all other possible alkylation products. In contrast, antibodies raised in chicken (α -hapten-IgY) recognized PNBM alkylated proteins whether or not they had been thiophosphorylated (Supplementary Fig. 1 online) and were not used in any further experiments. α -hapten-IgG comprised approximately 3% of total serum IgG, yielding milligrams of antibody for western blot and kinetic experiments. Meanwhile, we used cells from this rabbit to generate hybridomas. Forty eight hybridoma supernatants were immunoreactive to a BSA-hapten conjugate, and forty had modest to excellent specificity for thiophosphate esters over thioethers (Supplementary Fig. 2a online). Several of these clones immunoprecipitated affinity-tagged histone H1 (Supplementary Fig. 2b). Clones with the strongest signal also specifically recognized thiophosphate alkylation products in the context of a whole cell lysate (Supplementary Fig. 2c); we subcloned each of these hybridomas. We used the subclone with the strongest signal in these assays (51-8) for large-scale rabbit monoclonal antibody (RmAb) production.

Specificity and yield of PNBM alkylation

Proteins contain several functional groups that could potentially react with PNBM, and hinder automated database searching and identification of AS kinase substrates from mass spectrometric data. To determine the extent of PNBM alkylation of proteogenic amino acids, we thiophosphorylated and alkylated c-Jun-GST (as in Fig. 1c), digested it with trypsin and analyzed it by tandem mass spectrometry. After standard MASCOT-based sequencing-software analysis, c-Jun-GST was readily identified with 56% sequence coverage (Fig. 2a), indicating PNBM does not modify alcohol, amine or carboxylic acid functional groups. Manual sequencing of unassigned tandem mass spectra yielded peptides that contained alkylated cysteine and thiophosphorylated serine residues. We localized sites of cysteine alkylation by inspection of the y -ion series (Fig. 2b). Initial tandem mass spectrometry spectra of thiophosphate ester-containing peptides contained few sequence ions; lowering the collision-induced disassociation energy produced the more informative spectra (Fig. 2c). We observed a prominent 169-Da mass shift, corresponding to elimination of *p*-nitrobenzyl thiol, from the molecular ion, which produced an additional y -ion series.

To determine the yield of thiophosphate alkylation, we synthesized a model thiophosphopeptide and monitored PNBM alkylation by high-performance liquid chromatography (HPLC; Supplementary Fig. 3a–c online). The reaction was complete after a 1-h treatment with 2.5 mM PNBM. We observed only one product in the HPLC chromatogram, and mass spectrometric analysis confirmed this to be the monoalkylated peptide (Supplementary Fig. 3d).

Generality of ATP γ S use and antibody recognition

To determine the general applicability of this labeling strategy, we tested each of the reaction steps with a diverse set of kinase substrate pairs. Several kinases have been reported to use ATP γ S as a phosphodonor (for a partial list, see ref. 15), although often with a reduced catalytic rate, k_{cat} (ref. 16). However, the generality of thiophosphate transfer among kinases remains unclear. We screened 15 kinases for the ability to thiophosphorylate protein substrates, and all but two (GRK2 and JNK2; data not shown) accepted ATP γ S as a phosphodonor. We alkylated the thiophosphorylated substrates from the remaining 13 kinases with PNBM and analyzed them for antibody recognition in the context of different kinase consensus phosphorylation motifs¹⁷ (Figs. 3 and 4). The polyclonal antibodies recognized each labeled substrate regardless of the modified residue (serine, threonine or tyrosine) and the amino acid residues surrounding the site of phosphorylation. When the monoclonal antibody became available, we tested a diverse subset of labeled kinase substrates (PKC δ , JNK1, GSK3 β , Src and CK1), and in every case the 51-8 antibody selectively recognized the modified substrate (Supplementary Fig. 2).

A*TP γ S synthesis and use by AS kinases

A*TP γ S contains two permutations from the natural kinase substrate ATP: an N6 appendage and substitution of the terminal oxygen of the γ -phosphate with sulfur (Fig. 4a). The N6 modification was introduced to the adenosine riboside, followed by diphosphorylation¹⁸ to afford N6-alkylated ADPs. We synthesized A*TP γ S analogs by condensation of the N6-alkylated ADPs with an S-protected phosphodiester¹⁹, to ensure that the sulfur on the γ -phosphate is non-bridging. Previous work in our laboratory has demonstrated that N6-benzyl and N6-phenethyl ATP are consistently selected, from a diverse panel of N6-modified ATP analogs, as efficient substrates for AS kinases. Based on these data we synthesized N6-benzyl and N6-phenethyl ATP γ S.

We asked if these A*TP γ S analogs were bio-orthogonal (not accepted by wild-type enzymes) and if the unnatural analogs could be accepted by AS kinases. We tested wild type and AS alleles of kinases with diverse roles in cellular signaling for their ability to utilize ATP γ S and A*TP γ S as thiophosphate donors: PKC δ , a second messenger activated kinase²⁰, Cdc5, the yeast polo-kinase which regulates cell division²¹, and JNK1, a stress activated MAP kinase²². The assay for thiophosphorylation was based on alkylating the *in vitro* kinase reactions with PNBM, followed by western blot analysis (Fig. 4b). Notably, wild-type kinases accepted ATP γ S and could not utilize A*TP γ S analogs. Each of the AS kinases were able to utilize ATP γ S and one or both of the A*TP γ S analogs: AS PKC δ favored N6-benzyl ATP γ S, AS Cdc5 favored N6-phenethyl ATP γ S, and AS JNK1 used both analogs to a similar extent. Western blot analysis provides a rapid assessment of the preferred nucleotides but does not allow quantitative analysis of the thiophosphorylation reaction. To obtain relative kinetic parameters for unnatural A*TP γ S analog usage, we developed a disassociation-enhanced lanthanide fluorescence immunoassay (DELFI A)²³, using europium-labeled α -haptan-IgG (Supplementary Fig. 4a,b online). The AS kinases typically had a lower specificity (k_{cat}/K_m) for ATP γ S than the corresponding wild-type kinase, but we observed an increase in k_{cat}/K_m when the AS kinase used its preferred A*TP γ S analog (Supplementary Fig. 4c). In each case the magnitude of k_{cat}/K_m correlated with the intensity of the corresponding band on the western blot.

Immunoprecipitation and identification of Erk2 substrates

To explore the utility of this technique to affinity label and immunoprecipitate kinase substrates in a cellular context, we used cells expressing endogenous amounts of AS Erk2. We prepared mouse embryonic fibroblasts (MEFs) from 13.5-day-old embryos of *Erk1^{-/-}Erk2^{+/+}* and *Erk1^{-/-}Erk2^{AS/AS}* mice. Erk2 was immunoprecipitated from each of these cell lines and assayed with A*TP γ S analogs; N6-phenethyl ATP γ S was a preferred nucleotide substrate for AS Erk2 and was not accepted by wild-type Erk2 (data not shown). We optimized concentrations of A*TP γ S and GTP, a competitor for any general nucleotidases or nonspecific phosphotransferases, that yielded specifically labeled AS Erk2 substrates by adding varying concentrations of the nucleotides to digitonin-permeabilized MEFs. Alkylation and western blot analysis of cells permeabilized in the presence of 100 μ M A*TP γ S and 1 mM GTP revealed several proteins that were selectively labeled in the AS Erk2-expressing cells (Fig. 5a). Immunoprecipitation of these samples with the 51-8 monoclonal antibody yielded many specifically enriched proteins as determined by western blot analysis (Fig. 5b). Silver staining revealed a set of bands at 200 kDa (Fig. 5c). We cut the entire length of the gel (both wild-type and AS lanes) into approximately 1-mm slices and digested them with trypsin. Mass spectrometry analysis and database searching identified each of the high-molecular-weight bands as the nucleoporin translocated promoter region (Tpr), which is a reported Erk2 substrate¹¹. We sequenced forty-five peptides, corresponding to Tpr, from liquid chromatography–tandem mass spectrometry analysis of these gels bands, one of which is shown in Figure 5d. None of the other selectively immunoprecipitated substrates were present at a level sufficient for mass-spectrometric identification.

DISCUSSION

Presently 40 kinases have been rendered analog specific (Supplementary Table 1 online), and it is expected that most protein kinases will be amenable to this approach²⁴. The use of AS kinases in combination with bio-orthogonal ATP analogs has permitted the identification of several AS kinase substrates, yet the vast majority of such pairs remain to be identified. Here we report a unified technique for AS kinase substrate labeling and phosphoprotein affinity purification, which is applicable to all three common types of phosphorylation. Additionally, the suite of immunoassays described here allow for the detection, quantification and purification of wild-type kinase substrates.

Another advance toward our goal of physiologically relevant assays of individual kinases is the description of a method to label substrates in gently permeabilized cells expressing endogenous levels of an AS kinase, Erk2. Immunoprecipitation of these samples, prepared from a modest amount of material (15×10^6 cells), enriched a sufficient quantity of Tpr for mass-spectrometric identification. Presently we are extending this study to identify additional kinase substrates in tissues isolated from AS kinase-expressing mice. We are also exploring the possibility of immunoprecipitating modified peptides to identify the sites of phosphorylation; this approach recently has been applied to identify tyrosine phosphorylation sites²⁵.

High-affinity serine and threonine phosphospecific antibodies typically rely on residues or motifs²⁶ flanking the site of phosphorylation, whereas phosphotyrosine antibodies can recognize tyrosine phosphorylated sites independent of context²⁵. Using this semi-synthetic approach we were able to convert each of these phospho-amino acids into an epitope that can be recognized by a single antibody—a new concept in the area of modification-specific antibodies. A key feature of the epitope construction strategy is discrimination of closely related reaction products, that is, specificity for thioesters over thioethers. The ability to provide a second filter that distinguishes desired from undesired reaction products rather than relying on a single completely bio-orthogonal reaction, such as click chemistry, oxime formation or the

Staudinger ligation²⁷, may prove to be a general approach to broaden the scope of chemical functionalities and modification strategies used in bio-orthogonal tagging schemes.

METHODS

Antibody generation and purification

We conjugated hapten (Supplementary Fig. 5 online) to succinylated KLH, by reaction with *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide HCl (EDCI) and *N*-hydroxysulfosuccinimide (sulfo-NHS) overnight at pH 8. We purified the product on a PD-10 gel filtration column (Amersham) and used it to immunize rabbits (Epitomics) and chickens (Aves Labs). We purified polyclonal antibodies on an affinity column (diaminodipropyl amine gel; Pierce) containing immobilized hapten. After elution, we concentrated the antibodies using a 10-kDa molecular weight cut-off Amicon ultra centrifugal filter. For DELFIA, we labeled the antibodies with Eu³⁺ according the manufacturer's instructions (Perkin Elmer). Monoclonal antibodies were produced by Epitomics, and screened in enzyme-linked immunosorbent assay as well as western blot and immunoprecipitation assays as described in Supplementary Methods online.

A*TP γ S analog synthesis

The overall synthetic scheme is illustrated in Supplementary Figure 6 online. We converted N6-modified ribosides²⁸ to the N6-modified ADP molecules by reaction with POCl₃, followed by phosphoric acid and 1,8-diaza-bicyclo[5.4.0]undec-7-ene (DBU)¹⁸. We quenched the reactions with 0.1 M triethylammonium bicarbonate (TEAB) and purified the products with strong cation exchange chromatography (0.1 M to 1 M of TEAB) on a Hi-Prep QFF column (Amersham Biosciences). We pooled fractions containing the diphosphates, lyophilized them and analyzed them by mass spectrometry. Yields were 25–30%. We synthesized A*TP γ S analogs as described for ATP γ S¹⁹, and purified and analyzed them as described above for the ADP analogs. We obtained the lyophilized solids in 6–8% yield.

In vitro kinase reactions followed by antibody detection

We incubated kinases with their respective substrates in appropriate kinase buffers (see Supplementary Methods). For screens of analog preference and orthogonality using western blot analysis, we used ATP γ S or A*TP γ S analogs at a concentration of 1 mM. For kinetic measurements, ATP γ S or A*TP γ S analog concentration varied from 0.1 μ M to 250 μ M. We alkylated proteins with 2.5 mM PNBM for 2 h at room temperature (18–22 °C) and analyzed the products by western blotting or DELFIA. For western blotting, we diluted the antibodies 1:15,000 in TBS (pH 8.0) containing 0.5% Tween 20 (TBST) and 5% milk. We rocked the blots overnight at 4 °C, then incubated them with goat anti-IgG horseradish peroxidase (Promega) or rabbit anti-IgY horseradish peroxidase (Sigma), and imaged them (chemiluminescence on film).

Mice

All experiments involving live animals were approved by The University of California San Diego Institutional Animal Care and Use Committee (IACUC). We produced *Erk2*^{AS/AS} knock-in mice using standard techniques of gene targeting by homologous recombination. A 2.7 kb short arm including exon 2 and a 6.4 kb long arm containing exon 3 were cloned into plox Δ TK. We modified exon 3 by site-directed mutagenesis to change the CAG glutamine codon at position 102 to GGC glycine. This eliminated an *Eco*0109t restriction site that we used for screening. We sent the targeting vector to the University of California San Diego Cancer Center Transgenic facility to produce transfected R1 mouse embryonic stem cells. Two clones that tested positive for homologous recombination and retention of the codon mutation

were used to produce chimeric mice by blastocyst injection. Upon breeding, the targeted allele was transmitted to offspring and further transmitted at a Mendelian frequency.

We bred *Erk2*^{AS/AS} mice retaining the neomycin-resistance gene to EIIA-cre mice²⁹ and selected offspring for successful recombination. We then crossed *Erk2*^{AS/AS} mice with *Erk1*^{-/-} mice. We intercrossed the resulting mice and selected them for an *Erk1*^{-/-}*Erk2*^{+/+} or *Erk1*^{-/-}*Erk2*^{AS/AS} genotype. We derived embryonic fibroblasts as described in the Supplementary Methods.

Cell permeabilization and Erk2 substrate labeling

We trypsinized 15×10^6 wild-type or AS Erk2 MEFs cells, pelleted (200g at 4 °C) and resuspended them in DMEM to 5×10^6 cells/ml. We added phorbol 12-myristate 13-acetate (PMA) (20 ng/ml) and ionomycin (1 μM) for 5 min at 37 °C and then pelleted the cells. Permeabilization proceeded for 5 min on ice in 1× Dulbecco's phosphate buffered saline and 1× kinase buffer (Cell Signaling) containing complete protease inhibitor cocktail (Roche), phosphatase inhibitor cocktails I and II (Calbiochem) and 50 μg/ml digitonin (Sigma). We pelleted and resuspended cells in the same buffer but without digitonin, and with 100 μM *N*-phenethyl ATPγS and 1 mM GTP. The kinase reaction proceeded at 30 °C for 30 min with gentle rocking. We then pelleted and lysed the cells on ice for 15 min in 0.5 ml RIPA buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1.0% NP-40 and 0.1% SDS) containing 25 μM EDTA. We cleared the lysates by centrifugation, alkylated them and stored them at -80 °C.

Immunoprecipitation of Erk2 substrates with 51-8 antibody

We removed PNBMs, which potently inhibits immunoprecipitation, by size-exclusion chromatography. We equilibrated PD-10 columns with RIPA buffer, applied 0.5 ml of alkylated proteins to the column and eluted them with RIPA buffer containing protease inhibitors. We collected 0.5-ml fractions; we pooled the protein-containing fractions (7–9) and pre-cleared them with 100 μl of 50% rProtG agarose (Invitrogen) for 5 h at 4 °C. Meanwhile 20 μg of 51-8 RmAb was bound to 100 μl of 50% rProtG agarose in 1.0 ml of RIPA buffer containing 0.5 mg/ml BSA for 5 h at 4 °C. After removal of the beads from the lysate, we added the antibody-bound beads. Immunoprecipitation proceeded overnight at 4 °C, and then we washed the samples 4× with 1.0 ml of RIPA buffer. We eluted bound proteins with SDS-PAGE sample buffer and analyzed them by western blotting and silver staining.

Mass spectrometry

We excised bands from either a coomassie-stained gel (c-Jun-GST) or a silver-stained gel (Tpr), and subjected them to in-gel tryptic digestion. We separated peptides with nano-HPLC and sequenced them by tandem mass spectrometry on a QSTAR-XL (Applied Biosystems). The spectra were submitted to a Mascot search to identify unmodified peptides, and we manually sequenced PNBMs-modified peptides.

Additional methods

Detailed descriptions of all experiments are available in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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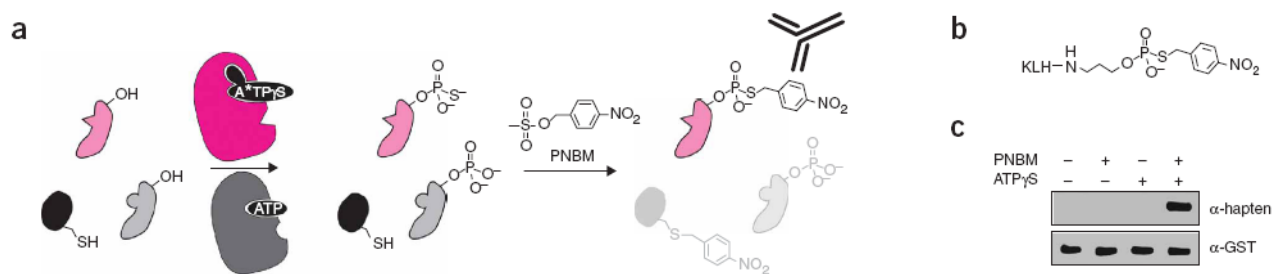


Figure 1.

Strategy for labeling individual kinase substrates. **(a)** Reaction sequence for affinity tagging AS kinase substrates. First, an AS kinase (magenta) uses N6-alkylated ATP γ S (A*TP γ S) to thiophosphorylate its substrates (pale magenta). In a second step, alkylation with PNBM yields thiophosphate esters and thioethers. Only AS kinase substrates are recognized by α -hapten-IgG. **(b)** Structure of the hapten conjugate used to elicit thiophosphate ester specific antibodies. **(c)** JNK1 was incubated with c-Jun-GST and combinations of ATP γ S and PNBM as indicated; reaction products were analyzed by western blot with α -hapten-IgG. α -GST-IgG confirms equal loading. Full-length blots are available in Supplementary Figure 1b.

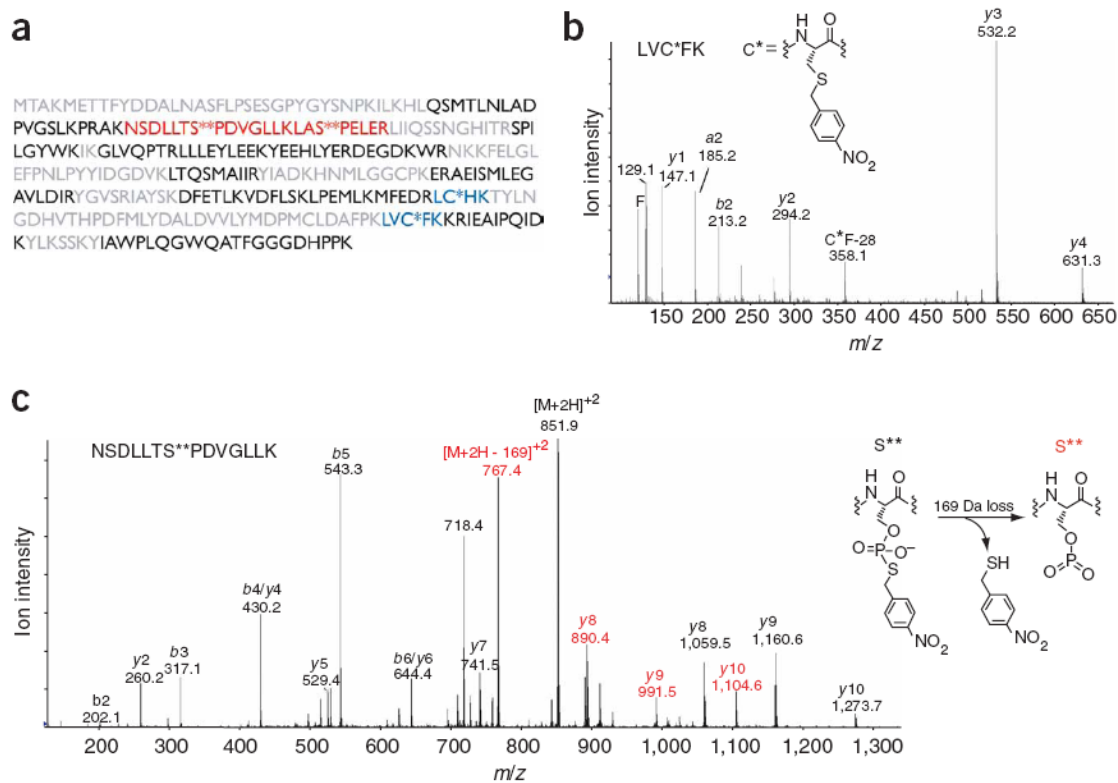


Figure 2.

Mass spectrometric analysis of thiophosphorylated and alkylated c-Jun–GST. **(a)** Sequence coverage of c-Jun–GST. Residues in black were identified by automated database searching of the unmodified peptides, blue indicates peptides that contain a modified cysteine, red indicates thiophosphate ester–containing peptides, and residues in gray were not observed. **(b)** Tandem mass spectrum of a thioether–containing peptide. C* indicates a nitrobenzyl–modified cysteine. **(c)** Tandem mass spectrum of a peptide containing a modified serine (S**), which corresponds to a site of JNK1 phosphorylation. A 169-Da loss, consistent with the depicted elimination, was observed from the doubly charged molecular ion and also gave rise to an additional y-ion series. Ions that have undergone this loss are labeled in red.

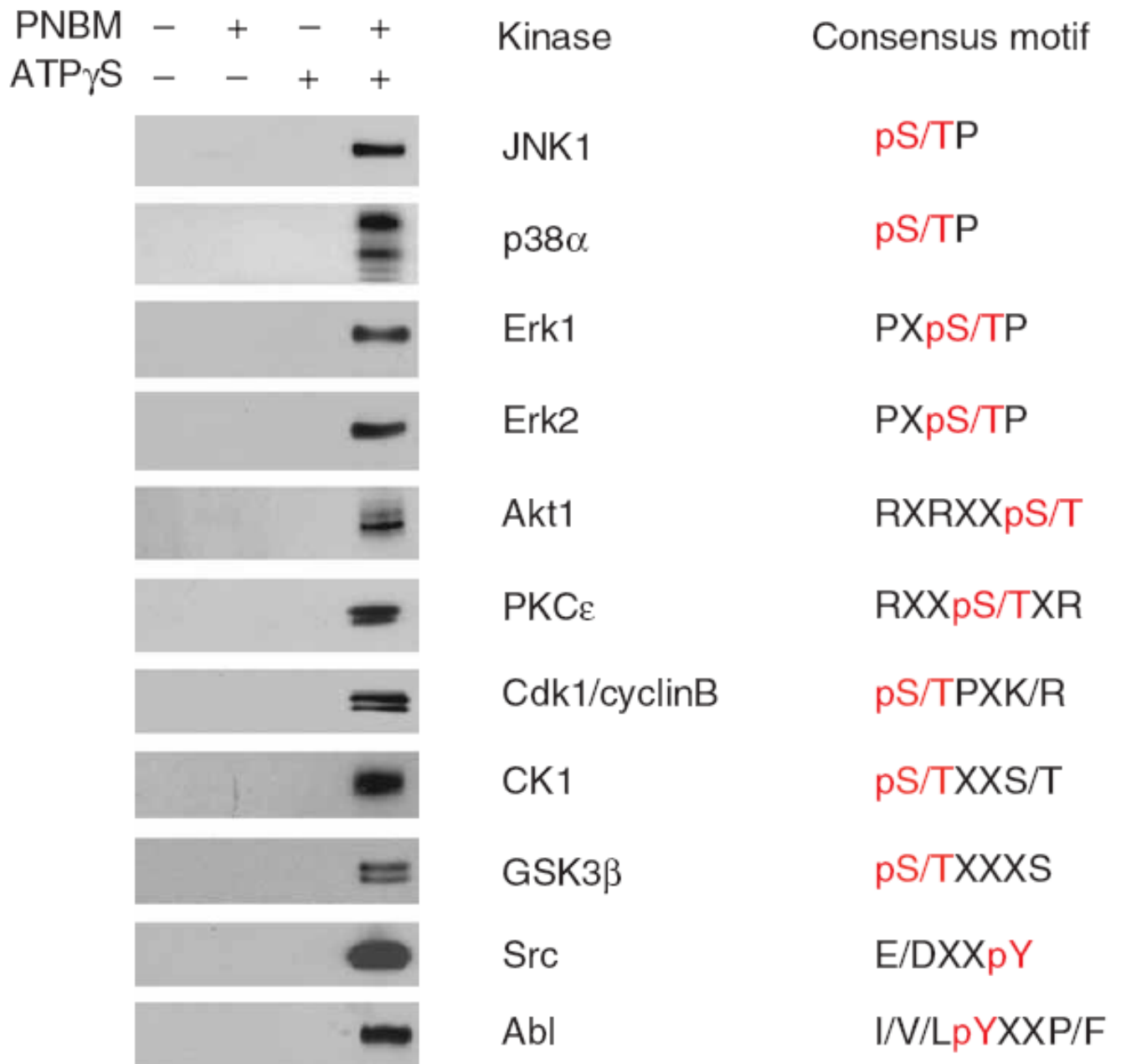


Figure 3. α -haptin-IgG detection of thiophosphate-esterified kinase substrates. Each blot is labeled with the corresponding kinase and its preferred phosphorylation consensus motif. A description of the conditions for each kinase reaction is available in the Supplementary Methods; full-length blots are available in Supplementary Figure 1c.

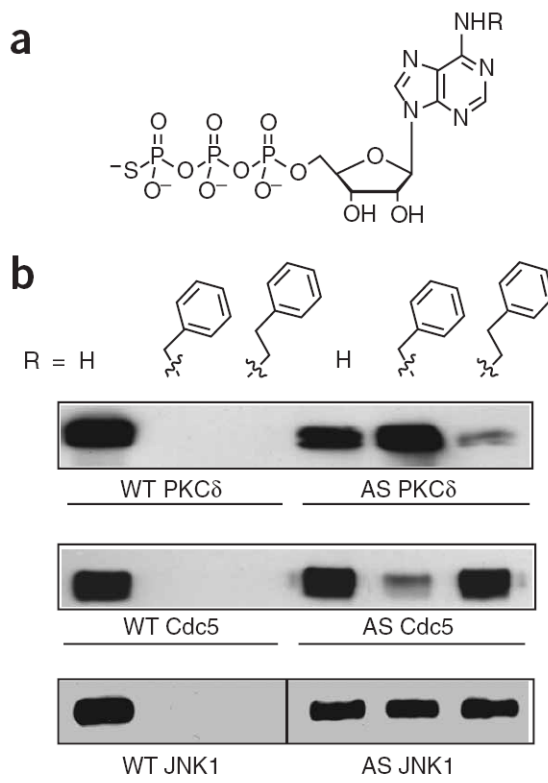


Figure 4. A*TP γ S analog orthogonality and acceptance by AS kinases. **(a)** Chemical structure of A*TP γ S, R indicates the site of N6 modification. **(b)** Kinase reactions with ATP γ S and A*TP γ S analogs. Following PNBM alkylation, the labeled substrates were detected with α -hapten-IgG western blot analysis, full-length blots are available in Supplementary Figure 1d. WT, wild type.

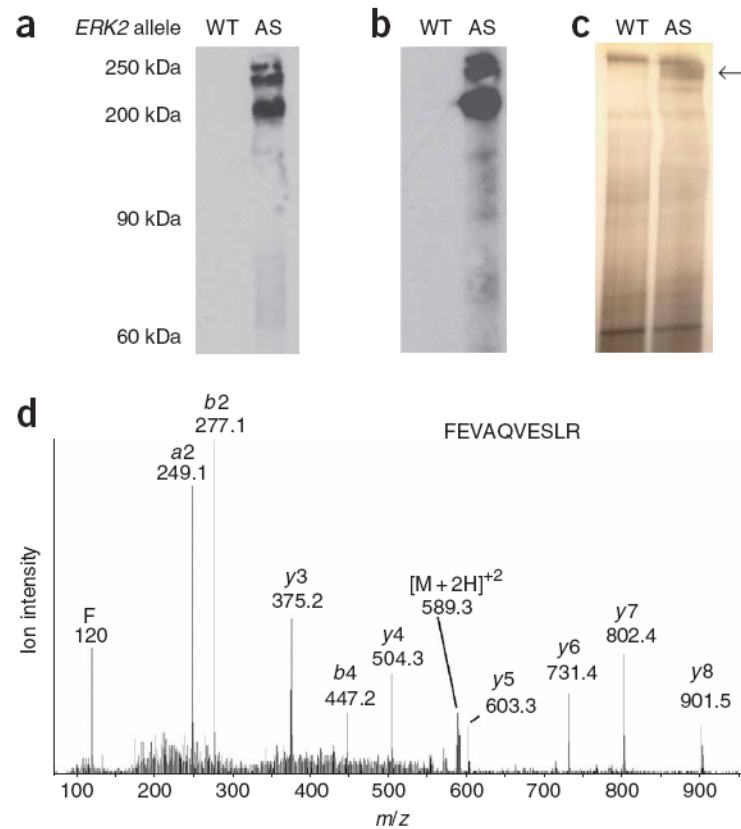


Figure 5. Labeling, immunoprecipitation and identification of AS kinase substrates. **(a)** MEFs expressing either wild-type (WT) or AS Erk2 were permeabilized, incubated with A*TP γ S, alkylated and analyzed by western blot (RmAb α -haptent-IgG). **(b)** Western blot analysis of the same samples as in **a** after immunoprecipitation. **(c)** Silver stain gel of immunoprecipitates in **b**. **(d)** Liquid chromatography–tandem mass spectrometry spectrum of a tryptic peptide, FEVAQVESLR, from the indicated band in **c**. Full-length blots and silver-stained gels are available in Supplementary Figure 1e.