Covalent capture of kinase-specific phosphopeptides reveals Cdk1-cyclin B substrates

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We describe a method for rapid identification of protein kinase substrates. Cdk1 was engineered to accept an ATP analog that allows it to uniquely label its substrates with a bio-orthogonal phosphate analog tag. A highly specific, covalent capture-and-release methodology was developed for rapid purification of tagged peptides derived from labeled substrate proteins. Application of this approach to the discovery of Cdk1-cyclin B substrates yielded identification of >70 substrates and phosphorylation sites. Many of these sites are known to be phosphorylated in vivo, but most of the proteins have not been characterized as Cdk1-cyclin B substrates. This approach has the potential to expand our understanding of kinase–substrate connections in signaling networks.

Results and Discussion
Chemistry-Based Purification of Thiophosphorylated Peptides. We and others have developed strategies for the capture of thiophosphorylated species (14, 15). These strategies rely on the nucleophility of the thiophosphate group, which can be modified by reaction with sulfur-selective electrophilic reagents. The key challenge is to distinguish between thiophosphate and other thiol nucleophiles, for example, cysteine. Given the abundance of cysteine, these groups must be distinguished with extremely high fidelity. The divergent ionization constants of cysteine and thiophosphate have been used to preferentially derivatize thiophosphate in the presence of cysteine. However, the moderate difference in pK<sub>a</sub> makes it very challenging to specifically isolate thiophosphate groups in the presence of a vast excess of cysteine nucleophiles, as in complex protein mixtures.

Here, we report a selective chemistry-based purification method

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Data Deposition: Raw MS data can be accessed at ftp://acbishop.ucsf.edu.

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substrates is digested to peptides, and the products are allowed to react with iodoacetyl-agarose. Thiol-containing groups react to form covalent bonds. Unbound specific tagging of the substrates of this kinase with a thiophosphate group. (B) Peptides are washed away. Thiophosphopeptides are specifically liberated by oxidation-promoted hydrolysis of the sulfur–phosphorus bond. The thiophosphoryl sulfur atom is replaced with oxygen in this step.

Fig. 1. Tagging and purification of kinase-specific phosphorylation sites. (A) A kinase is engineered to accept an ATP analog bearing a substitution at the N6 amine by mutation of the conserved “Gatekeeper” residue (as-kinase, for analog-sensitive). Replacement of a γ-phosphoryl oxygen with sulfur gives the ATP analog $N^\alpha$-(benzyl)ATP–γ-S, which bears a transferable thiophosphate group. A kinase reaction performed with an as-kinase and $N^\alpha$-(benzyl)ATP–γ-S results in specific tagging of the substrates of this kinase with a thiophosphate group. (B) Thiophosphopeptide purification. A protein mixture containing tagged substrates is digested to peptides, and the products are allowed to react with iodoacetyl-agarose. Thiol-containing groups react to form covalent bonds. Unbound peptides are washed away. Thiophosphopeptides are specifically liberated by oxidation-promoted hydrolysis of the sulfur–phosphorus bond. The thiophosphoryl sulfur atom is replaced with oxygen in this step.

Specific Labeling of Cdk Substrates by Using $N^\alpha$-(benzyl)ATP–γ-S. The most stringent test of an affinity enrichment method for substrate identification is its application to a kinase with hundreds of low-abundance substrates. We chose the Cdks as a test case because of the wide variety of critical cell-cycle events they coordinate and the likelihood that many of their substrates would be difficult to detect without enrichment.

Human Cdk–cyclin complexes bearing the analog-sensitizing mutation F80G (as-Cdks) are capable of using a radiolabeled version of our ATP analog [N(6)-(benzyl)ATP–γ-35S] to label endogenous substrates in cell extracts with high specificity (Fig. 3A). In the absence of as-kinase, background labeling was very low, indicating minimal usage of the analog by endogenous enzymes. Many of the substrates are labeled by individual Cdk–cyclin complexes, or shared between pairs of complexes, indicating substrate specificity contributions from the Cdk and the cyclin partner (21). High-resolution mapping using 2D gels revealed labeling of hundreds of proteins by Cdk1-cyclin B (Fig. 3B). To determine which Cdk1-cyclin B substrates were abundant enough to be identified by analysis of excised gel spots, the silver-stained 2D gels were digitized and overlaid on the autoradiographic data (SI Fig. 7). We found minimal correspondence between the radiolabeled and silver-stained species, indicating labeled isoforms were present at mostly subnanogram levels, below the limits of protein identification methods. These experiments validate Cdk1-cyclinB as a stringent test case for any kinase substrate identification method because the number of direct substrates is large, and their varied and often low abundance is a challenge to traditional separation methodologies.

Identification of Cdk1-Cyclin B Substrates and Their Phosphorylation Sites in Cell Extracts. We next pursued identification of Cdk1-cyclinB substrates in HeLa cell extracts. To reduce sample complexity, we separated the lysates into five fractions by ammonium sulfate fractionation. Purified recombinant as-Cdk1-cyclin B and N(6)-(benzyl)ATP–γ-S were added to cell lysate fractions, and then thiophosphopeptides produced by trypsinolysis were specifically recovered by using our purification methodology. The recovered peptides were analyzed by nanosecond liquid chromatography coupled to a Q TRAP tandem mass spectrometer. Peptide fragmentation data were interpreted by using Mascot (22) and verified by manual inspection. Searches considered oxidative modifications. Consistent with expectation, we observed oxidation of methionine to the sulfoxide, rarely to the sulfone, but we never observed the natural form. We never observed peptides containing reduced or oxidized cysteine residues or oxidation of amines or other residues. Although the substantial majority of recovered peptides bore proline-directed phosphorylations consistent with labeling by Cdks,
we also recovered five phosphopeptides representing six non-proline-directed sites in four nucleotide-binding enzymes, one of which is known to autophosphorylate by using our ATP analogs (SI Table 2). We also recovered unmodified peptides from 20, mostly highly abundant, proteins (SI Table 3). Eighty-five percent of these were recovered from a single purification, suggesting an incomplete wash step.

In total, 72 proline-directed phosphorylation sites were identified (Fig. 4 and SI Table 4). The identified proteins are involved in a wide range of cellular functions from cell cycle control to receptor signal transduction. Many of the proteins we identified are known Cdk substrates with biological roles for their phosphorylation, confirming that our methodology can rapidly uncover significant biological connections. Examples of proteins in this class include protein phosphatase 1α (23), DNA ligase I (24), RNA polymerase II (25), lamins A/C (26) and B (27), stathmin (28), and multiple nucleolar components. In protein phosphatase 1α, which antagonizes mitotic Cdk1 activity by dephosphorylating its substrates, the site we found (T320) is known to be phosphorylated by Cdk1-cyclin B in mitosis, resulting in inhibition of phosphatase activity and thus promotion of the mitotic state (23). In lamin A/C we detected phosphorylation of serine 22 and serine 392, sites required for nuclear envelope disassembly in mitosis (26, 29).

Analysis of the functions of the candidate substrates revealed that many are associated with processes or structures known to be regulated by Cdks. For example, we recovered the known Cdk substrates nucleophosmin and nucleolin, nucleolar components involved in production of ribosomes. We also found three sites in the candidate substrate, Treacher Collins syndrome protein (Treacle), a nucleolar protein involved in ribosomal DNA gene transcription (30). Similarly, we uncovered four proteins involved in nucleotide biosynthesis; two are known Cdk substrates and two are candidate substrates. These results indicate that our discovery method is capable of rapidly identifying biologically relevant connections between the target kinase and specific phosphorylation sites in its substrates.

Further evidence for the relevance of the candidate Cdk sites comes from the observation that many are phosphorylated in vivo. We compared our results with several large phosphoproteomic datasets (6–8). Interestingly, at least 36 of our sites, or 50%, are known to be phosphorylated in vivo in HeLa cells. At least four additional sites are known to be phosphorylated in mouse homologs (9). Our results therefore provide an important link between these sites and a specific kinase, and strongly suggest that our method is capable of uncovering relevant in vivo connections between a kinase and its substrates.
In Vitro Validation of Selected Candidate Substrates. To further confirm that our methodology targets normal Cdk1 substrates and does not alter the kinase’s substrate phosphorylation preferences, we examined the ability of four of the identified proteins to act as substrates for wild-type Cdk1 and natural ATP. These proteins were produced in bacteria, purified, and phosphorylated with Cdk1-cyclin B and ATP. After digestion with trypsin, phosphopeptides were enriched by using immobilized iron-affinity chromatography and analyzed by LC-MS/MS. In every case, we observed phosphorylation of the same site seen in our original substrate discovery experiments, including one site matching only a minimal Cdk consensus (Table 1 and SI Fig. 8). These results indicate that the engineered kinase/nucleotide pair Cdk1F80G/N6B-H9253 displays the same substrate phosphorylation site preferences as wild-type Cdk1/ATP.

Many Phosphorylation Sites Identified Lack an Optimal Cdk Consensus Sequence. Peptide phosphorylation experiments have shown that an optimal Cdk phosphorylation consensus sequence consists of a target serine or threonine followed by a proline, with an arginine or lysine residue in the +1 position ([ST]Px[KR]) (4, 31). We found that 68% of the phosphorylation sites we identified (49 of 72) occurred in full consensus sequences, whereas the remaining 32% fell within suboptimal sites containing only the minimal necessary proline feature. Considering only those sites “known in vivo,” we found approximately the same distribution, with 39% of sites (16 of 41) matching only the minimal proline-directed consensus. The abundance of suboptimal sites highlights the challenge inherent in assigning kinase–substrate pairings based on phosphoproteomic data alone; none of these sites could be inferred to be Cdk1 sites.

Table 1. Cdk1–cyclin B phosphorylation site mapping in four purified recombinant substrates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Site from extracts</th>
<th>Site(s) mapped in purified protein</th>
<th>Site sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drp1/Dnm1</td>
<td>S616</td>
<td>S616</td>
<td>SPQK</td>
</tr>
<tr>
<td>HuR</td>
<td>S202</td>
<td>S202</td>
<td>SPAR</td>
</tr>
<tr>
<td>hnRNP-k</td>
<td>S216</td>
<td>S216</td>
<td>SPK</td>
</tr>
<tr>
<td>TPX2</td>
<td>S738</td>
<td>S738, S59, T72, T338, S486</td>
<td>SPKF</td>
</tr>
</tbody>
</table>

Wild-type Cdk1-cyclin B was found to phosphorylate the same sites uncovered in our screen, and several additional sites in the protein Tpx2.
based solely on sequence analysis. Given the large number of proline-directed kinases, it’s tempting to speculate that such sites may be substrates for multiple kinases, perhaps serving as signal integrators. Indeed, even optimal sites may serve such a function. For example, we found an optimal site in hnRNP-K (S216) that was determined to be phosphorylated by JNK (32). Phosphorylation of this site along with S53 was found to increase the transcriptional activity of hnRNP from AP1-bearing promoters. We speculate that Cdk1-cyclin B activity may similarly regulate this protein.

Identification of Cdk1-Cyclin B Substrates in Purified Nuclear Envelope. We next sought to evaluate the utility of our method for the discovery of kinase substrates in purified subcellular compartments. This approach serves to greatly enrich a subset of the proteome while preserving the structural context of the enriched proteins. We chose to focus on the nuclear envelope. Mitotic disassembly of the nuclear lamina and of the nuclear pore complex (NPC) are known to be imposed in large part by Cdk activity (33, 34).

Rat liver nuclei were prepared as described in ref. 35 and nuclear envelope was prepared by DNase/RNase digestion followed by heparin treatment as described in ref. 36. This material contains the nuclear membrane, transmembrane and tightly associated proteins, and the NPC. Purified recombinant as-Cdk1-cyclin B and NβB-γ-S were added to the preparation; three experiments were performed and recovered phosphopeptides were analyzed by LC-MS/MS.

We recovered 20 phosphorylation sites on 11 proteins (Fig. 5). Confirming identifications in the larger screen, we identified known Cdk1 phosphorylation sites in the nuclear lamina constituents lamin A/C and B. We also identified sites on three transmembrane proteins that associate directly with the lamin network: Lap1, Lap2B, and MAN1. Lap1 and Lap2 are believed to be involved in assembly of the nuclear lamina, and mitotic phosphorylation of Lap2B is known to abrogate binding to lamin B and to chromosomes (reviewed in ref. 34). We also identified phosphorylation sites within constituents of the NPC (Nup53, Nup133, and Nup358) and in the nucleoplasmic domains of the three transmembrane proteins (gp210, POM121, and NDC1) that assemble and/or anchor the NPC into the nuclear membrane (37). Nup53 is known to tightly associate with the nuclear envelope and lamin B and may bind to the NPC assembly protein NDC1 (37, 38). It is also the vertebrate ortholog of yeast Nup53p, a known Cdk1/Cdc28 target (39). These results indicate that we were able to recapitulate discovery of known Cdk1 targets in the nuclear envelope and to discover candidate targets known to be regulated by mitotic phosphorylation.

Advantages and Limitations of the Method. Although our substrate discovery technology allowed us to uncover numerous known Cdk1 substrates and candidates in our initial screen, it also missed a number of known substrates, in particular, low-abundance proteins such as transcription factors. Because our model thiophosphopeptide experiments indicate that our purification process can recover a target peptide from a 500,000-fold excess of background, we believe these false negatives result primarily from the broad dynamic range of cellular proteins, as well as difficulty in achieving high-stoichiometry phosphorylation in cell extracts. The comprehensive identification of substrates is therefore likely to require measures such as an increase in experimental scale, further fractionation of extracts, or increased focus on purified subcellular compartments.

Tagged peptides with cysteine residues are not recoverable with our method. To assess the resulting loss of coverage, we conducted in silico digests of the human proteome and asked what fraction of the resulting peptides contained cysteine. For relevance to mass spectrometric analysis, we considered only those peptides between 7 and 25 residues in length. For trypsin, 24.5% of the peptides contained cysteine (SI Table 5). In practice this loss could be largely mitigated by conducting parallel digests with two or more proteases.

Hydrolysis of the thiophosphate linkages to the beads converts these peptides to ordinary phosphopeptides. Although our enrichment technique is extremely specific for tagged peptides, it would be ideal to have a unique identifier that remains covalently attached to the phosphopeptide beyond this step. One way of accomplishing this is to conduct the hydrolytic release of the captured tagged peptides in 32O-labeled water (demonstrated in SI Fig. 9), which results in the liberated phosphopeptide bearing an isotopically labeled phosphate. Alternatively, a nucleotide analog bearing one or two isotopically labeled oxygen atoms in the transferred thiophosphate could be used (SI Fig. 10).

We have recently reported a method for identification of askinase targets by using a monoclonal antibody that recognizes a chemically derivatized thiophosphorylation adduct (14, 43). Although the antibody-based approach does not immediately identify the phosphorylation site targeted by an askinase, it is complementary to the method reported here because it leads to purification of the entire substrate protein. This allows multiple substrate tryptic peptides from the same protein to be analyzed, and is not influenced by the presence of cysteines near the phosphorylation site.

The capture-and-release kinase substrate identification method provides a means of rapidly determining connections between an individual kinase and candidate substrates, at amino acid level resolution. Critically, candidates can be identified in extremely complex protein mixtures that preserve important features of biological context. The method provides a potentially powerful strategy for deciphering the connections between individual protein kinases and their substrates in signaling networks, and provides a complementary approach to large-scale phosphoproteomic studies.

Materials and Methods

Analog-Sensitive Kinase Preparation. Purified analog-sensitive (F80G) Cdk-cyclin complexes were produced by using a baculovirus expression system as described in ref. 40.

Fig. 5. A schematic depiction of the nuclear envelope and embedded nuclear pore complex, indicating the phosphorylation sites recovered in our experiments (not to scale). The indicated positions of the phosphorylation sites (red circles) are approximate.
Synthesis of N\(^6\)-(Benzyl)ATP-\(\gamma\)-S-Phosphorothioate. The synthesis of this reagent follows the procedure described for ATP-\(\gamma\)-S by Goody and Eckstein (1941) with the exception of chromatographic separations (SI Fig. 11). In brief, sodium thiosphosphate (Sigma) was protected at sulfur with 3-chloropropionamide. The product was activated with diphenyl phosphorochloridate and coupled to N\(^6\)-(benzy)ADP. The protected product was isolated by ion-exchange chromatography. Treatment with 0.1 M sodium hydroxide at 100°C for 10 min gave the deprotected species. The product was again isolated by ion-exchange chromatography. Mass and structure were verified by MS/MS analysis.

Synthesis of N\(^6\)-(Benzyl)ATP-\(\gamma\)-S-35S-Phosphorothioate. N\(^6\)-(Benzyl)ATP was converted to N\(^6\)-(benzy)ATP-\(\gamma\)-S\(^{35}\)S by using an enzymatic system as described in ref. 13.

Autoradiographic Analysis of CdK Substrates. For 1D analysis, 20 μg of total HeLa cell extract were labeled by the addition of 5 μM of N\(^6\)-(benzy)ATP-\(\gamma\)-S\(^{35}\)S, 1 mM magnesium chloride, and either buffer or purified recombinant as-Cdk–cyclin complexes (200 ng), at room temperature for 30 min. The samples were resolved by PAGE on 7.5–15% gradient gels, dried, and imaged by using a Typhoon system (GE Healthcare). For 2D analysis of as-Cdk1-cyclin B substrates, the reaction was scaled 10-fold, and proteins were separated over an immobilized pH 3–10 gradient by using an IPIGPhor system (Amersham Biosciences). Separated proteins were resolved by SDS/PAGE on a 7.5–15% gel.

Model Thiosphopeptide Studies. Purified, bacterially expressed Fin1 protein was labeled by incubation with Cdk2-cyclin A, ATP-\(\gamma\)-S, and 5 mM MgCl\(_2\), then digested with trypsin. Ten picomoles of digest were added to 100 μl of iodoacetamide (BioLink gel, Pierce) in 100 μl of 50% acetonitrile, alone or with 10 μg of Jurkat cell extract digest. The beads were incubated overnight in the dark with rotation, and then loaded in a 1-ml disposable column. The flow-through fraction from the Fin1-only sample was set aside, and the beads were washed with 10 ml each of water, 5 M NaCl, 50% acetonitrile, and 5% formic acid in water. The beads were treated with 500 μl of a 1 mg/ml solution of Oxone. Eluting phosphopeptides were concentrated by capture on 1 μl of Poros R3 resin (Applied Biosystems) immobilized in a gel-loading pipette tip, and eluted directly onto MALDI plate by using a standard DHB MALDI matrix solution, for analysis with a PROTOF instrument (PerkinElmer).

Preparation of HeLa Cell Total Cell Extract. Pelleted cells were washed in the National Cell Culture Center (NCCC) and resuspended in hypotonic lysis buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, plus protease inhibitors) and lyzed by douncing. Nuclei and membranous fractions were pelleted by centrifugation and resuspended in lysis buffer containing 500 mM KCl. After centrifugation, the supernatant was added to the initial lysate.

Preparation of Nuclear Envelope. Rat liver nuclei were prepared as described in ref. 35 and stored at ~8°C in 100-U (3 × 10\(^6\) nuclei) aliquots. Nuclear envelope was prepared as described in ref. 36.

Purification of Cdk1-cyclin B Substrate-Derived Phosphopeptides from HeLa Cell Extracts. Ammonium sulfate was added to HeLa cell total extract and proteins precipitating at 25%, 40%, 50%, 60%, and 75% of saturation were separated by centrifugation and stored frozen as pellets until needed. Pellets were thoroughly dialyzed against HEPES-buffered saline before further use (20 mM HEPES, pH 7.4, 150 mM NaCl). Extract fractions (0.1–1 mg) were labeled by addition of purified recombinant as-Cdk1-cyclin B to 1% mass of total protein, 1 mM N\(^6\)-(benzy)ATP-\(\gamma\)-S-35S-phosphorothioate, and 5 mM MgCl\(_2\). After 30 min of labeling, the reactions were stopped by addition of EDTA and then digested with trypsin. Thiosphopeptides were recovered by using our purification methodology, as described for the model thiosphopeptide experiments. The recovered peptides were analyzed by nanoscale LC-MS/MS. Proteins and phosphorylation sites identified over the course of several experiments were combined in a Microsoft Access database.

LC-MS/MS Analysis. Phosphopeptide samples purified from HeLa extract fractions were analyzed by nanoscale liquid chromatography coupled on-line to a Q Trap tandem mass spectrometer (Sciex). The peptides were separated over the course of 100 min by using a nonlinear gradient of 5% to 30% acetonitrile containing 0.1% formic acid, at a flow rate of 150 nl/min. A 75 μm i.d. by 15 cm RP column (LC Packings) was used to resolve peptides, and an upstream trap column was used to facilitate sample concentration and transfer from the autosampler. Survey scans were performed by using the “Enhanced MultiCharge” mode. Neutral loss survey scans targeting loss of phosphate were occasionally used. Fragmentation spectra were acquired automatically in information-dependent acquisition (IDA) mode and interpreted both manually and with MASCOT (Matrix Science).

Database Searching. MS data files were searched against the Uniprot and NCBInr databases by using MASCOT. First-pass searches considered one missed cut and oxidation of methionine to the sulfone or sulfone, and phosphorylation of serine or threonine. Further searches considered two missed cuts, the presence of three potential glycosylation sites and glycosylation, and two peptide bonds. Peptides with MASCOT expect scores of >0.05 were included; most of the reported hits had substantially better scores (SI Tables 2–4).

In Vitro Phosphorylation Assays. ORFs encoding Drp1 and TPX2 were purchased as Ultimate ORF Gateway vectors (Invitrogen) and shuttled into the bacterial expression vector pDEST17 by site-specific recombination. pDEST17 encodes an in-frame N-terminal 6x His tag. ORFs encoding HuR and hnRNP-K were purchased as CNAs (Invitrogen) and cloned into pDONR221 by PCR-mediated incorporation of terminal recombination sequences, followed by recombination. The ORFs were then shuttled into pDEST17 by recombination. All four proteins were expressed in Escherichia coli strain BL21(DE3)pLysS (Novagen), and purified by IDA-Cobalt affinity chromatography. Five micrograms of each purified protein were labeled with 20 ng of Cdk1-cyclin B and 100 mM ATP for 30 min, then reactions were stopped with 5 mM EDTA and digested. Phosphopeptides were enriched by using immobilized iron (Phos-Select beads; Sigma), eluted with EDTA, and analyzed by nanoscale LC-MS/MS using the QTrap instrument.

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