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A Chemical Genetic Approach for the Identification of Direct Substrates of Protein Kinases

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1. Introduction

Protein kinases form one of the largest superfamily of enzymes that play pivotal roles in controlling almost every signaling pathway ***(1)***. Deregulated kinase activity thus leads to multiple diseases, including various forms of cancer ***(2)***, inflammatory and autoimmune diseases ***(3)***, neurodegenerative diseases ***(4,5)***, diabetes ***(6)***, and HIV infection (***(7)***). Signaling networks regulated by kinases are complex and highly interconnected. Additionally, many kinases display overlapping substrate specificities in vitro and can functionally compensate for each other in single gene knockout experiments ***(8,9)***. Therefore, unraveling of these pathways to dissect the role of any particular kinase (normal or oncogenic) has remained one of the major challenges ever since the first kinase was purified. Ideally, if the substrate of each kinase could be identified in a cell, it would provide a baseline for understanding the complex cellular functions and consequently also provide a blueprint for novel targets for drug discovery.

To date, multiple strategies for kinase substrate identification have been developed. Screening of degenerate peptide ***(10)***, cDNA ***(11–13)***, and phage display expression libraries ***(14)*** have proven to be valuable for the identification of several putative targets of multiple purified kinases. Recently, Zhu et al., ***(15)*** used nanowell chip technology to analyze the kinase activities of 119 yeast protein kinases with 17 different substrates, revealing novel substrate specificities. Phosphorylation of the proteome chips expressing all the yeast proteins with different purified kinases is potentially a powerful tool to spatially resolve kinase substrate specificity at the proteome level ***(16)***.

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The key determinant of kinase substrate specificity in vivo however, depends on cellular localization and protein–protein interactions, hard to recapitulate in an in vitro system ***(17)***. As a result, various techniques have been used to dissect the precise substrate phosphorylation events in signaling cascades. Recently, an approach has been described for the simultaneous measurement of multiple active kinase states using polychromatic flow cytometry, allowing the identification of distinct signaling cascades in various cellular processes ***(18)***. This analysis at a single cell level allows monitoring of signaling mechanisms in rare cell populations. However, the requirement of phosphospecific antibodies for detection, limits the efficacy of this method to the known substrates only in a signaling cascade. Other techniques for kinase substrates identification include immunoprecipitation of relevant kinase complexes ***(19,20)***, mass spectrometry and functional proteomics ***(21,22)***, and modulation of divalent metal requirements in cell lysates ***(23)***. Although, the fundamental question of which kinase is responsible for a given phosphorylation event remains elusive because of the presence of many kinases in the same pathway that exhibit overlapping substrate specificities ***(24)*** and because all kinases use ATP as the phosphodonor substrate.

Recently, we have developed a chemical genetic approach to address this question ***(25–27)***. This review will focus on advances in this chemical genetic approach for identification of direct substrates of multiple protein kinases. A kinase of interest is engineered to accept a non-natural phosphate donor substrate (A*TP) that is poorly accepted by wild-type protein kinases in the cell. The modification of the kinase's active site, so that it accepts a structurally modified γ-^{32}P-labeled nucleotide analog provides a unique handle by which the direct substrates of any particular kinase can be traced in the presence of other protein kinases for the first time (**Fig. 1**). The design strategy uses the engineering of a unique active site pocket in the ATP binding site of the target kinase and a complementary substituent on the A*TP analog. This unnatural pocket is created by the replacement of a conserved bulky residue with a glycine or an alanine in the active site and the complementing substituent on ATP is created by attaching bulky substituents at the N-6 position of ATP (ex: N^6-(benzyl) ATP, N^6-(phenethyl) ATP etc., **refs. *26,28***).

To confer highly specific sensitivity of the engineered kinase to unnatural ATP analogs, engineered amino acid residue should meet following design criteria.

- The wild-type residue should have a conserved bulky side chain such that mutation to a smaller residue creates a new pocket in the active site.
- The residue should be highly conserved across the whole kinase family in terms of similarity of side chain volume at the minimum.

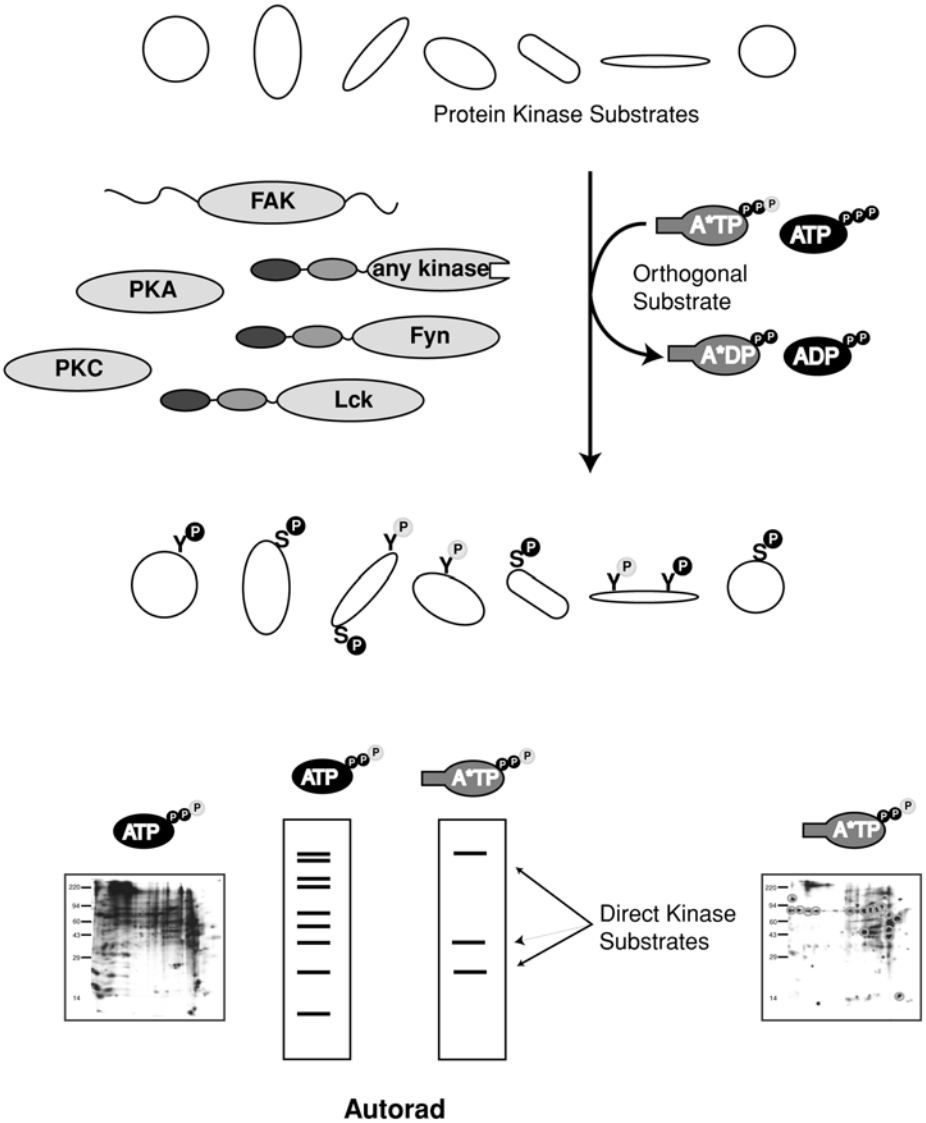

Fig. 1. Schematic representation of the use of A*TP by an engineered kinase. The use of specific phosphodonor substrate (A*TP) for one kinase allows for its direct substrates to be uniquely radiolabeled (grey P represents ^{32}P). S indicates serine and Y indicates tyrosine. Ovals represent catalytic or regulatory domains (black = Src-homology 3 domain, dark grey = Src-homology 2 domain, light grey = catalytic domain).

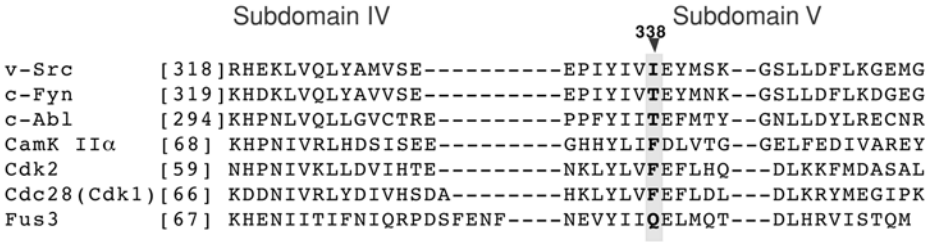

Fig. 2. Kinase domain sequence conservation and partial sequence alignment of the protein kinases. The highly conserved "gatekeeper" residue is shown in bold.

- Mutation of the residue should be functionally silent and should not dysregulate the kinase or alter its phosphoacceptor specificity.
- Ideally the mutation should be generalizable to all members of the protein kinase superfamily (est. 500 human kinases)

A functionally conserved residue was identified in the V subdomain of the kinase active site (I338 in v-Src) that could be mutated to an alanine or a glycine, thus creating a new pocket in the active site (**Fig. 2**). Further studies revealed that this conserved large residue in fact functions as a "gatekeeper" by blocking an existing pocket in the kinase active site. Thus, by clipping the bulky side chain of the key residue, we uncover a "path" for the bulky N^6-substituents on ATP analogs to gain access to the existing pocket rather than engineering a whole new pocket ***(27)***. The distinctive importance of the gatekeeper residue has also been well exploited for designing specific inhibitors for both wild-type serine/threonine and tyrosine kinases ***(29)***. Some kinases require an additional sensitizing mutation immediately amino-terminal to the conserved DFG motif in kinase subdomain VII.

This key residue makes close contact with ATP and often has a smaller side chain. However, several kinases have large amino acids at this position that interfer with the binding of ATP analog. Mutation of this residue to an alanine or a glycine effectively allows efficient catalysis with A*TP analogs ***(30,31)***. The engineered kinase fulfills several necessary criteria to uniquely tag its authentic substrates in the presence of all wild-type kinases. Engineered kinase:

- Accepts an ATP analog (A*TP) that is a poor substrate for all wild-type protein kinases;
- Uses A*TP with high catalytic efficiency;
- Exhibits reduced catalytic efficiency for the natural nucleotide substrate (ATP) so that, in the presence of cellular levels of ATP (~5 m*M*), the engineered kinase preferentially uses A*TP as the phosphodonor.

And finally all of these criteria are met by creating a functionally silent mutation generalizable across the entire kinase superfamily. Structural and functional assessment of peptide specificity of mutant protein kinases with orthogonal ATP analogs showed that the creation of a unique nucleotide binding pocket does not alter the phospho-acceptor binding site of the kinase ***(32)***. A panel of optimal peptide substrates of defined sequence, as well as a degenerate peptide library was utilized to assess the phospho-acceptor specificity of the engineered "traceable" kinases. The specificity profiles for the mutant kinases were found to be identical to those of their wild-type counterparts, further confirming that the engineered mutation is indeed functionally silent.

This strategy has recently been applied to identify the direct targets of two different serine/threonine kinases in cellular extracts: c-Jun amino terminal kinase (JNK; **ref. *31***) and CDK2 ***(33)***. Addition of immunopurified mutant JNK (JNK analog specific-3 [JNK-as3]) and [γ-^{32}P]N^6-(phenethyl) ATP analog into whole cell extract resulted in the specific phosphorylation of hnRNP-K protein. This study further confirmed hnRNP-K as a true physiological target of JNK by in vivo ^{32}P labeling, whose phosphorylation at serine 216 and serine 353 by JNK increases its transcriptional effects. In another study, phosphorylation by baculovirus expressed mutant CDK2/cyclins (CDK2-as1) and A*TP revealed Kaposi's Sarcoma-Associated Herpesvirus K-bZIP protein as its direct substrate in BCBL-1 cell extracts. This is the first report that shows K-bZIP protein as a direct target of CDK2, suggesting its potential involvement in cell cycle.

Most recently, we applied this approach to look for the direct substrates of v-Src in fibroblasts ***(34)***. v-Src-induced transformation of fibroblasts leads to the tyrosine phosphorylation of more than 50 proteins; however, it is not clear if all of these proteins are direct v-Src substrates or if they are phosphorylated as a consequence of the v-Src activation of other kinases. Therefore, a global analysis of direct v-Src substrates was carried out by the addition of bacterially expressed mutant XD4 (constitutively active form of Src, consisting mainly SH1 domain) and [γ-^{32}P]N^6-(benzyl) ATP into NIH3T3 cell lysate. Interestingly, no substrate phosphorylation was observed suggesting that may be the lack of SH2/SH3 domains in XD4 prevented it to form relevant complexes. However, the addition of immunopurified mutant v-Src (v-Src-as1) also showed minimal phosphorylation of substrates in cell lysate. Interestingly, when mutant v-Src was endogenously expressed in NIH3T3 cells, addition of [γ-^{32}P]N^6-(benzyl) ATP into the cell lysate showed phosphorylation of more than 20 proteins on a 2D gel (**Fig. 3**). As a control, addition of radiolabeled A*TP analog to wild-type v-Src expressing cell lysate showed no phosphorylation. Mass spectral analysis of several of these spots revealed novel substrates

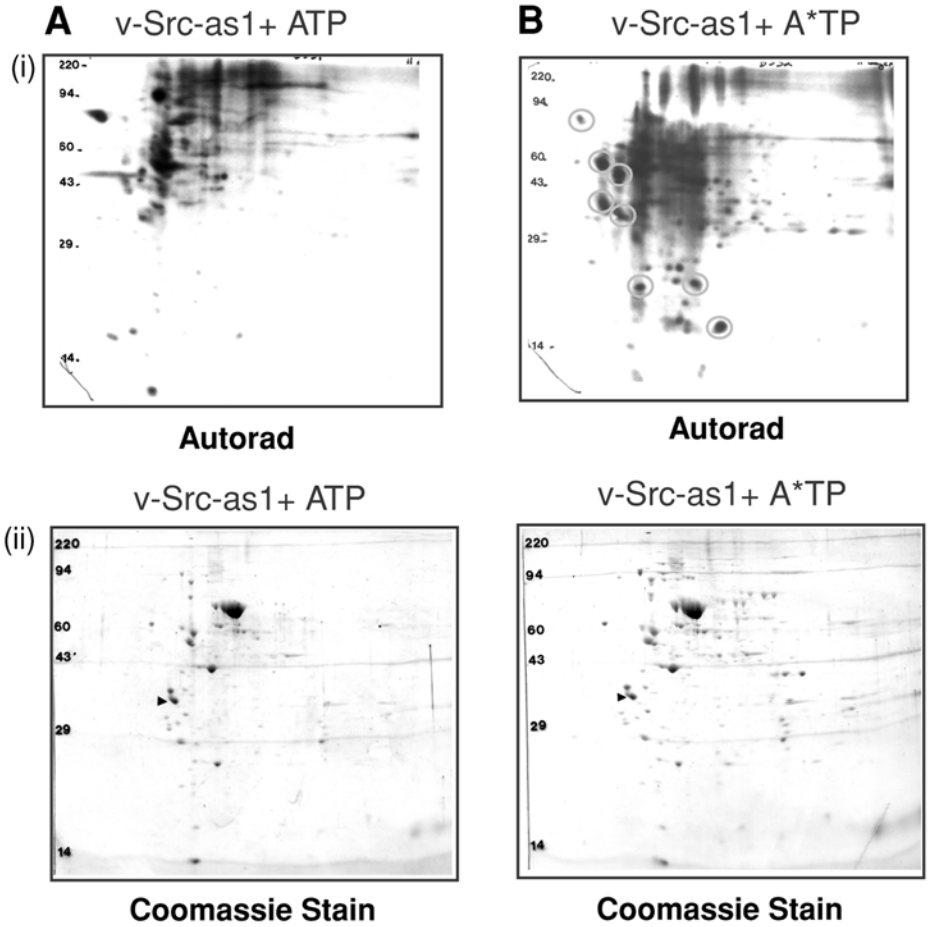

Fig. 3. Differential phosphorylation of direct v-Src substrates in *v-Src-as1* NIH3T3 cell lysate with [γ-^{32}P]N^6(benzyl)ATP. **(A)** 2D gel electrophoresis of kinase reaction of *v-Src-as1* NIH3T3 cell lysate and [γ-^{32}P]ATP in the presence of 100 μ*M* ATP for 45 min. Internal standard (tropomyosin) is shown as an arrow at MW 33 K and pI 5.2. (i) Autorad; (ii) The gels were Coomassie blue stained, dried, and scanned. **(B)** 2D gel electrophoresis of kinase reaction of v-Src-as1 NIH3T3 cell lysate and [γ-^{32}P]A*TP in the presence of 100 μM ATP for 45 min. (i) Autorad; (ii) The gels were Coomassie blue stained, dried, and scanned.

of v-Src. This result strongly suggests that kinases anchored in proper signaling scaffolds best phosphorylate their physiological substrate proteins and added kinases (especially tyrosine kinases) can't easily access the relevant complexes.

This result further prompted us to look for substrates of v-Src in different signaling complexes. Additionally, we predicted that use of v-Src immunoprecipitation to enrich for v-Src substrates would allow the identification of substrates present at low concentration and those substrates that are phosphorylated at low stoichiometry. We isolated multiple complexes (anti-v-Src, -Fak, -cortactin) in which v-Src was directly responsible for phosphorylation of all proteins in the complex as might be expected for this highly active oncoprotein. Interestingly, a very low abundance scaffolding protein Dok-1 was found to be a direct substrate of v-Src in all of these complexes. Yet, in one particular complex isolated by immunoprecipitation with an anti-RasGAP antibody, both v-Src and Dok-1 were present yet v-Src was only responsible for a subset of the phosphorylation products, and not of Dok-1. This result suggested that although v-Src was present and active in multiple complexes, it is not a promiscuous kinase that always phosphorylates a given protein (Dok-1) in all signaling complexes. Other kinases in these complexes are responsible for highly specific phosphorylation events, revealing such protein complexes to be more dynamic and specifically regulated than previously appreciated.

Further studies revealed a number of surprising features of v-Src substrates that contradict currently accepted model of tyrosine kinase specificity. For example, analysis of the individual tyrosine phosphorylation sites on the multiply phosphorylated protein Dok-1 (Dok-1 had 14 potential tyrosine phosphorylation sites) revealed that out of four phosphorylation sites, only two (Y361 and Y450) were definitively sites phosphorylated by v-Src, even though three (Y295, Y314, and Y361) of the four sites shared a similar consensus sequence (YXXP). Moreover, the site that was predicted to provide a nonoptimal v-Src phosphorylation site, was one of the sequences directly phosphorylated (L$\mathbf{Y}_{\mathbf{450}}$SQV). One of the v-Src phosphorylation sites (Y361) on Dok-1 produces an optimal binding site for at least two different SH2 containing proteins, v-Src itself and RasGAP. Because the other v-Src phosphorylation site Y450 on Dok-1 is not an optimal consensus phosphorylation site for Src, its phosphorylation may require proper binding of v-Src to Dok-1. Thus, stable association of v-Src and Dok-1 may allow v-Src to phosphorylate the suboptimal v-Src consensus site, which in turn creates a binding site for the SH2 domain of Csk. Such a processive phosphorylation model has been proposed by Zhou and Cantley ***(24)*** and by Scott and Miller ***(35)***. Because both Src and rasGAP compete for binding at pY361, it is likely that bound rasGAP blocks the binding of v-Src at pY361, resulting in the observed failure of v-Src to phosphorylate Dok-1 in rasGAP complex. In summary, we found that v-Src phosphorylation of substrates can be regulated at the level of protein complex formation and that a limited subset of sites of heavily phosphorylated proteins are directly phosphorylated by a single kinase, v-Src. It further suggests that

identification of direct phosphorylation sites solely based on the optimal sequence specificity of a particular kinase may be misleading.

The role of Dok-1 phosphorylation by v-Src revealed a model for the assembly of negative regulatory proteins (rasGAP and Csk) onto the scaffolding protein Dok-1. Further studies revealed that Csk negatively regulates the duration of c-Src signaling in a feedback mechanism. Because *v-Src* lacks this critical phosphorylation site, it is not responsive to this arm of the retrograde signaling apparatus, contributing to its potent transformation. Mutation of v-Src phosphorylation sites Y295 or Y361 (rasGAP binding sites) abrogated the inhibitory effect of Dok-1 on cellular transformation, indicating that these sites are necessary for the repression of transformation activity, thereby highlighting yet another arm of retrograde signaling that is controlled by v-Src itself. Identification of the direct substrates of v-Src leads to a model for the precise order of assembly of a retrograde signaling pathway in v-Src transformed cells and has provided a new insight into the balance between those signals that promote cell transformation mediated by v-Src catalyzed tyrosine phosphorylation and those that inhibit it.

This chemical genetic approach has also been used in the identification of substrates in multiple cell lines in response to different stimuli and in various tissues. Addition of any particular analog sensitive serine/threonine kinase to fractionated tissue homogenate results in specific phosphorylation of their direct substrates. This method offers the advantage of looking at the differential phosphorylation pattern of any kinase of interest in normal versus diseased tissue type. The direct radiolabeled targets thus obtained can be further confirmed by in vivo labeling or by phosphotyrosine immunoblotting (Kim et al., unpublished results).

More recently, we have designed novel ATP analogs with enhanced selectivity for mutant kinases ***(36)***. These ATP analogs are more orthogonal than previously reported analogs and should allow the detection of protein substrates too scarce to be detected previously ***(34)***. However, intact cells are the best context to identify bona fide kinase substrates, because subcellular localization and protein-protein interactions are powerful determinants of kinase signaling specificity ***(17)***. To accomplish this goal, we recently engineered a kinase that accepts an unnatural triphosphate analog, but is insensitive to cellular milieu of ATP (~5 m*M*). N^4-(benzyl) AICAR triphosphate is orthogonal to wild type kinases and is a substrate of T106G p38. Thus, generation of a unique kinase/phosphodonor pair that is insensitive to ATP will provide a higher signal to noise ratio of substrate labeling, thereby allowing the identification of low abundance kinase substrates in intact cells (Ulrich et al., unpublished results).

In addition to using a chemical genetic approach for substrate identification, we have also applied our strategy to probe the role of individual kinases by using

allele-specific orthogonal inhibitors ***(37,38)***. By using the same analog-sensitive mutant of a particular kinase together with the chemically modified "bumped" inhibitor, we have recently shown the role of three kinases in yeast: Cdc28p ***(39)***, Cla4p ***(30)***, and Pho85p ***(40)***. This approach is extremely powerful because it allows elucidation of the role of any kinase of interest in vivo.

2. Materials

1. 6-Chloropurine riboside, benzylamine, carbonyl diimidazole, trimethyl phosphate and $POCl_3$ (Aldrich) were stored at room temperature under anhydrous conditions.
2. 1 *M* TEAB buffer was prepared by suspending 1 mole of triethylamine (Aldrich) in water in a total volume of 1000 mL, followed by CO_2 bubbling until a pH of 7.5 was attained.
3. Diethylaminoethyl-Sephadex (DEAE-Sephadix A1-25) and protein A Sepharose (Pharmacia) were soaked in water overnight and washed several times with water before use.
4. Luria Bertani (LB) Superbroth, isopropyl β-D-thiogalactopyranoside (IPTG), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), glutathione S transferase (GST) beads, and glutathione (Sigma or any other supplier).
5. Optimal peptide substrate for the kinase of interest can be purchased if commercially available or can be custom synthesized.
6. [γ-^{32}P]ATP and [γ-^{32}P]H_3PO_4 (NEN or any other supplier).
7. p81 filter and polyvinylidene membrane (Millipore).
8. GFP protein-based kinase substrates were generated as published previously ***(41)***.
9. 4G10 (Upstate) and HRP-conjugated anti-mouse antibody (VWR cat. no. 55550).
10. Supersignal kit (Pierce).
11. Kodak Biomax MS film, Kodak X-OMAT AR film and Biomax MS intensifying screen (Kodak).
12. Strong anion exchange column (Rainin).

3. Methods

The methods described below outline (1) the steps for chemical genetic dissection of a kinase signaling cascade and (2) experimental procedures for synthesis of ATP analogs, protein expression, screening of ATP analogs, and determination of direct substrates in cellular systems.

3.1. Steps for Chemical Genetic Dissection of a Kinase Signaling Cascade

3.1.1. Identify Site of Mutation

The "gatekeeper" and the other key residue immediately amino-terminal to the conserved DFG motif in kinase subdomain VII can be readily identified by kinase catalytic domain sequence alignment of a desired kinase using Kinase Sequence Database located at http://kinase.ucsf.edu/ksd. This database

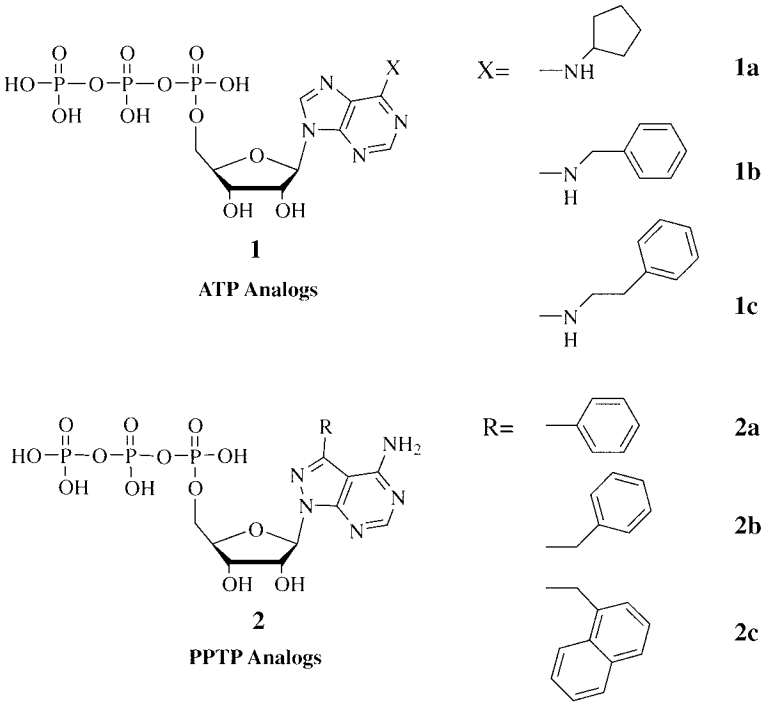

Fig. 4. Structure of ATP and PPTP (pyrazolopyrimidine triphosphate) analogs.

contains information on 290 protein kinase families for a total of 5041 protein kinases from more than 100 organisms ***(42)***.

3.1.2. Construction of Analog-Sensitive Kinase Allele

1. For most kinases, mutation of the "gatekeeper" amino acid residue in the ATP binding site (equivalent to I338 in v-Src) to either a glycine (analog sensitive v-Src-1, v-Src-as1) or an alanine (v-Src-as2) renders them sensitive to orthogonal ATP analogs. Additional mutation may be required of the residue flanking the amino terminal of DFG motif to an alanine (as-3) for those kinases that have a bulky side chain on this residue.
2. Recombinant-engineered kinase is expressed as a fusion protein in *Escherichia coli*, insect cells, or mammalian cells following precedents with the corresponding wild-type kinase.

3.1.3. Determining the Optimal ATP Analog for the Engineered Kinase

1. A panel of cold ATP analogs (**Fig. 4**) are tested as inhibitors of [γ-^{32}P] ATP-dependent phosphorylation of an optimal peptide substrate by the engineered kinase in an in vitro kinase reaction.

2. For assay of catalytic activity, the engineered tyrosine kinase is tested with ATP analogs and highly efficient GFP protein based kinase substrates may be used, or alternatively any known phosphoacceptor substrate of the kinase of interest ***(41)***.
3. The ATP analog with the highest catalytic efficiency paired with the desired engineered kinase are chosen.

3.1.4. Choice of Cell System

1. Recombinant-engineered kinase is added exogenously to the desired cell lysate or tissue extracts for an unbiased analysis of direct substrates of the kinase of interest.
2. Alternatively, engineered kinase is expressed endogenously in appropriate cell type. This approach is particularly useful for tyrosine kinases that require proper SH2/SH3 interactions for substrate phosphorylation (see above).

3.1.5. Optimize Kinase Reaction Conditions

1. The kinase reaction is performed with appropriate radioactive [γ-^{32}P]A*TP in the presence of cold ATP. Because the unnatural ATP analog is orthogonal to wild-type kinases, cold ATP is added to the kinase reaction to maintain proper stoichiometry of phosphorylation. Therefore, amount of radioactive A*TP/cold ATP is empirically determined.
2. Reaction time (10 s–20 min) should be empirically optimized.
3. Optimal cell lysis buffer conditions are determined to maximize kinase activity and thus substrate phosphorylation ***(43)***.

3.1.6. 2D Gels or Immunoprecipitation

1. Substrate phosphorylation is conducted in different immune complexes containing the engineered kinase. This method allows for the enrichment of potential substrates and thus can be resolved completely by 1D polyacrylamide gel electrophoresis (PAGE) ***(34)***.
2. For the identification of substrates in total cell lysate, phosphorylated substrates are further concentrated by immunoprecipitating with phosphospecific antibodies or by other affinity resins and then separated on 1D PAGE (Shokat et al., unpublished results).
3. Alternatively, phosphorylated substrates from total cell lysate are also isolated by 2D PAGE ***(34)***

3.1.7. Mass Spectral Analysis or Immunoblotting

1. Isolated radiolabeled substrates are identified by mass spectral analysis. The only limitation of this method is for the substrates that are present in low abundance.
2. Direct substrates of a kinase can also be identified by immunoblotting or by double immunoprecitation.

3.1.8. Confirmation In Vivo

1. Substrates identified by in vitro kinase assay are further confirmed in an in vivo system. For confirming the substrates of oncogenic-engineered tyrosine kinase, substrates are immunoprecitated from the transformed cells and immunoblotted with a phosphotyrosine-specific antibody ***(34)***. These substrates can be further confirmed by using allele-specific orthogonal inhibitors, which should inhibit the phosphorylation event catalyzed by the engineered kinase only.
2. The engineered kinase can also be activated appropriately in the cell and the substrates are confirmed similarly.
3. For confirming the substrates of serine/threonine kinases, cells are labeled in vivo with ^{32}P phosphoric acid and substrates are isolated after stimulation and checked on by autoradiography for the incorporation of phosphate. These substrates are also further confirmed by using allele specific inhibitors (Shah et al., unpublished results).

3.1.9. Functional Studies

1. Depending on the signaling pathways controlled by the kinase of interest, the role of phosphorylation of novel direct substrates are explored.
2. On the other hand, identification of novel substrates also leads to the identification of novel pathways.

3.2. Experimental Procedures

3.2.1. Synthesis of [γ-^{32}P]N^6(benzyl) ATP ([γ-^{32}P]A*TP)

1. N^6(benzyl)adenosine was synthesized by refluxing 6-chloropurine riboside (1 mmol) (Aldrich) with benzylamine (5 mmol) in ethanol (10 mL) overnight. Ethanol was removed in vacuo and the resulting oily residue obtained was crystallized from ethanol (yield 90%). N^6 (benzyl) ADP was synthesized by sequential phosphorylation according the method of Hecht and Kozarich ***(44)***. To an ice-cooled suspension of N^6(benzyl) adenosine (68 mg, 0.2 mmol) in trimethyl phosphate (0.5 mmol), $POCl_3$ (0.025 mL) was added and the reaction mixture was stirred at 0°C for 1 h, after which the reaction was quenched with 5 mL of 1 *M* triethylammonium bicarbonate (TEAB buffer, pH 7.5). Solvent was removed in vacuo at <40°C by rotary evaporation. The resulting slurry was purified on DEAE (A-25) Sephadex (Pharmacia) column using TEAB pH 7.5 (0.1–0.5 *M* gradient). The purified N^6(benzyl) AMP shows a retention time of 7.5 min on a strong anion exchange high-performance liquid chromatography column (SAX, cat. no. 83-E03-ETI, Varian) using a gradient of 5–750 m*M* ammonium phosphate, pH 3.9, in 10 min at a flow rate of 0.5 mL/min.
2. In the second step, a solution of N^6(benzyl) AMP (44 mg, 0.1 mmol) and carbonyl diimidazole (81 mg, 0.5 mmol) in dimethylformamide (DMF) (5 mL) was stirred at room temperature for 20 h, after which methanol (35 μL) was added. After 1 h, a solution of tributyl ammonium phosphate (1 mmol) was added in DMF (1 mL).

The reaction was stirred for additional 24 h. After quenching the reaction mixture with 2 mL of TEAB buffer (pH 7.5), solvent was removed in vacuo at <40°C and the residue was purified as described above (retention time 9.7 min).

3. N^6(benzyl) ADP (2.5 μmol, molar absorbancy (ε_{max}) 15.4×10^3 at 265 nm at pH 7.00) was dissolved in DMF (200 μL) and carbonyl diimidazole (8 mg, 10 μmol) was added to it. The reaction mixture was stirred for 24 h at room temperature after which methanol (4 μL) was added and the reaction was stirred for an additional 1 h. ^{32}P orthophosphoric acid (5 mCi, 8500 Ci/mmol) was dried in vacuo, dissolved in DMF (100 μL), and was added to the reaction mixture. After stirring for 24 h, DMF was removed in vacuo and the radiolabeled analog was purified by ion-exchange chromatography using DEAE (1.5- × 7-cm packed volume) and a gradient of 0.1–1 *M* TEAB buffer pH 7.5 at a flow rate of 1.5 mL/min. The purified product was concentrated in vacuo at <40°C by rotary evaporation. The concentrated triphosphate was redissolved in 200 μL of water and the concentration was determined by scintillation counting (yield 20%). The [γ-^{32}P] N^6(benzyl) ATP was characterized by co-injection of the radiolabeled material with an authentic sample of N^6(benzyl) ATP ***(25)*** on a strong anion exchange-high-performance liquid chromatography column (retention time 11.2 min).

3.2.2. Expression of Wild-Type and Mutant Kinase in E. coli *(GST-XD4 as a Model System)*

XD4 is a truncated form of v-Src, that contains an intact SH1 catalytic domain, but lacks SH2 and SH3 domains and exhibits higher specific activity than full length v-Src ***(45)***.

1. Expression of wild-type and mutant XD4 was carried out in DH5α cells. A single colony was inoculated into 25 mL of LB superbroth liquid media with 100 μg/mL ampicillin. The culture was grown at 37°C overnight. This culture was added to 250 mL of LB superbroth. After 2 h at 37°C (OD_{600} = 0.5), IPTG was added to a final concentration of 1 m*M*. The culture was shaken for 5 to 6 h at 37°C and then was centrifuged at 2500*g* for 15 min.
2. The pellet was resuspended in 5 mL of 25 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT buffer, and lysed twice at 8000 psi in a French press at 4°C.
3. The lysate was centrifuged again at 2500*g* for 10 min. The supernatant was added to 1 mL of reconstituted glutathione beads and was gently shaken for 30 min on ice.
4. The slurry was added to a column (Fisher Scientific cat. no. 11-387-50) and the beads were washed with 10 mL of 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA buffer followed by 50 m*M* Tris-HCl pH 8.0 (5 mL). The glutathione fusion protein was eluted with 4 mL of 10 m*M* free glutathione, 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl solution.

3.2.3. Kinase Assays with GST-XD4 to Determine Optimal ATP Analog

3.2.3.1. Inhibition Assay

1. Purified XD4 and XD4-as1 proteins were mixed with kinase buffer, 250 μ*M* Src peptide substrate (EIYGEFKKK), [γ-^{32}P]ATP (5 μCi) and 100 μ*M* cold A*TP analog in a total volume of 30 μL.
2. After 20 min incubation at room temperature, the reaction was terminated by the addition of 50 μL of 10% phosphoric acid.
3. After a brief centrifugation, 50 μL of each supernatant was spotted on p81 filters, washed four times with 1% phosphoric acid, rinsed with acetone, and finally radioactivity remaining on the filters was determined.

3.2.3.2. Catalytic Activity Assay

1. Kinase assays were performed using GFP protein-based kinase substrates ***(41)*** with both wild-type and mutant XD4 in the presence of cold ATP analogs for 20 min at room temperature.
2. The reaction mixtures were boiled in the sample buffer for 5 min. Proteins were resolved on 12% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and were probed with anti-phosphotyrosine (4G10), followed by horseradish peroxidase conjugated anti-mouse antibody. Enhanced chemiluminescence was used for antibody detection as described in the manufacturer's instructions and visualized by Supersignal kit.

3.2.4. Transfection and Retroviral Infection of v-Src and v-Src-as1 into NIH3T3 Cells

1. Both *v-Src* and *v-Src-as1* (cloned into pBabe puro plasmids) were transiently transfected into Bosc 23 cells using the calcium phosphate transfection method ***(46)***.
2. Culture medium containing the retroviruses were harvested from 72 h to 5 d after transfection. Viral supernatants were stored at –80°C.
3. Negative control retroviruses lacking *v-Src* were prepared by using pBabe puro vector alone.
4. 5×10^5 NIH3T3 cells were seeded on a 10-cm culture dish and were allowed to attach to the plate for several hours. Infection was performed by adding the retrovirus supernatant (3 mL) in the presence of polybrene (4 μg/mL).
5. After 3 h the viral supernatant was removed and replaced with fresh media (10% bovine calf serum/Dulbecco's modified eagles medium). After 48 h, the infected cells were selected in the presence of 2.5 μg/mL of puromycin. After another 24 h greater than 95% cells were viable by trypan blue staining.
6. The expression of wild-type and *v-Src-as1* in NIH3T3 cells was confirmed by immunoprecipitation and immunoblotting. Both wild-type and v-Src-as1 proteins were present in equal amounts.

3.2.5. Kinase Assays and 2D Gel Electrophoresis of v-Src and v-Src-as1 NIH3T3 Cells

1. Cells were lysed at 4°C in modified RIPA buffer (1% NP 40, 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.25% sodium deoxycholate, 2 m*M* ethylenebis-(oxyethylenenitrilo)tetraacetic acid, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) and cleared by centrifugation at 20,000*g* for 15 min at 4°C.
2. Cleared lysates were mixed with 10 $MgCl_2$, 10 m*M* $MnCl_2$, 1 m*M* sodium orthovanadate, 100 μ*M* ATP, 5 μCi of [γ-^{32}P] ATP (6000 Ci/mmol) or [γ-^{32}P]N^6 (benzyl) ATP (8500 Ci/mmol) and were incubated for 45 min at room temperature.
3. Cell lysates were boiled for 5 min in sample buffer (62.5 m*M* Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 2.5 mg/mL DTT, 2.5% β-mercaptoethanol) and separated on 10% SDS-PAGE gels. Separated proteins were electrophoretically transferred either to immobilon polyvinylidene membranes with a semidry apparatus (Owl Scientific) for 1 h at 10V. For autoradiography, the membranes were exposed to Kodak Biomax MS film (Kodak) with a Biomax MS intensifying screen at –70°C.
4. 2D gel electrophoresis of radiolabeled samples was conducted by Kendrick Labs (Madison, WI) using 2% pH 3.5–10 ampholines (Pharmacia) for 9600 volt h. One mg of an IEF internal standard, tropomyosin, was added to each sample. 10% SDS slab electrophoresis was performed for about 4 h at 12.5 mA/gel. The gels were Coomasassie blue stained, dried, and exposed to Kodak X-OMAT AR film.

3.2.6. Immunoprecipitation and Kinase Assays of v-Src and v-Src-as1 NIH3T3 Cells

1. Cells were lysed at 4°C in modified RIPA buffer with 0.1% SDS and 2 m*M* sodium orthovanadate.
2. After centrifugation, the cleared lysate was incubated with 1–5 μg of the desired antibody at 4°C for 2 h with gentle rotation, followed by incubation with 50 μL of 50% protein A Sepharose slurry.
3. The immune complexes were washed three times (500 μL each) in modified RIPA buffer without SDS and twice in kinase buffer (30 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, and 10 m*M* $MnCl_2$).
4. Kinase reactions were initiated by adding 0.5 μCi of [γ-^{32}P]ATP alone or 1 μCi of [γ-^{32}P]N^6(benzyl) ATP in the presence of 10 μ*M* ATP and incubated for 10–15 min (as indicated in figure legends) at room temperature.
5. After boiling for 5 min in sample buffer immune complex proteins were separated on 10% SDS-PAGE gels, electrophoretically transferred and exposed as described before.

3.2.6. Mass Spectral Analysis

1. Gel spots were digested by adding 0.05 μg of modified trypsin in the minimum amount of 0.025 *M* Tris-HCl, pH 8.5, and leaving the gel overnight at 32°C.
2. Peptides are extracted with 2 × 50 μL of 50% acetonitrile/2% TFA and the combined extracts are dried and resuspended in matrix solution prepared by making a 10 mg/mL solution of 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile/0.1% TFA and adding two internal standards, angiotensin and bovine insulin to the matrix solution.
3. Matrix-assisted laser desorption/ionisation mass spectrometric analysis was performed on the digest using a PerSeptive Voyager DE-RP mass spectrometer in the linear mode. These peptides were analyzed by peptide mass fingerprinting and data base searching using Protein Prospector (Columbia University/HHMI, Protein Core Facility, New York, NY 10032).

4. Notes

1. A*TP are unstable at room temperature and susceptible to freeze/thaw. Therefore, it is best to make small aliquots of A*TP solution in water and freeze them at –20°C.
2. Optimal storage conditions (temperature and buffers) for the kinase of interest should be determined to retain maximal activity over a period of time.
3. 1 *M* TEAB buffer is best when prepared fresh, however, it can be stored at 4°C for couple of days.

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