Engineering Src family protein kinases with unnatural nucleotide specificity

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Background: Protein kinases play a central role in controlling diverse signal transduction pathways in all cells. The identification of the direct cellular substrates of individual protein kinases remains the key challenge in the field.

Results: We describe the protein engineering of v-Src to produce a kinase which preferentially uses an ATP analog, N^6-(benzyl) ATP, as a substrate, rather than the natural v-Src substrate, ATP. The sidechain of a single residue (Ile338) controls specificity for N^6-substituted ATP analogs in the binding pocket of v-Src. Elimination of this sidechain by mutation to glycine produces a v-Src kinase which preferentially utilizes N^6-(benzyl) ATP as a phosphodonor substrate. Our engineering strategy is generally applicable to the Src family kinases: mutation of the corresponding residue (Thr339 to glycine) in the Fyn kinase confers specificity for N^6-(benzyl) ATP on Fyn.

Conclusions: The v-Src tyrosine kinase has been engineered to exhibit specificity for an unnatural ATP analog, N6-(benzyl) ATP, even in a cellular context where high concentrations of natural ATP are present (1–5 mM), where preferential use of the ATP analog by the mutant kinase is essential. The mutant v-Src transfers phosphate more efficiently with the designed unnatural analog than with ATP. As the identical mutation in the Src-family kinase Fyn confers on Fyn the ability to recognize the same unnatural ATP analog, our strategy is likely to be generally applicable to other protein kinases and may help to identify the direct targets of specific kinases.

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Key words: ATP analog, protein engineering, protein phosphorylation, Src protein kinase

Received: **23 October 1997**
Revisions requested: **2 December 1997**
Revisions received: **6 January 1998**
Accepted: **8 January 1998**

Published: **15 February 1998**

Chemistry & Biology February 1998, **5**:91–101
http://biomednet.com/elecref/1074552100500091

Introduction

Protein kinases catalyze the transfer of the γ phosphate from ATP to serine, threonine, tyrosine, or histidine residues of protein substrates. They are found in many cellular compartments, in membranes, the cytosol, associated with the cytoskeleton or in the nucleus, and are involved in a wide variety of cellular functions, including cytokine responses, antigen-dependent immune responses, regulation of the cell cycle, modification of cell morphology, learning and memory, ion-channel regulation and stress responses (to ultraviolet light and oxidants) [1]. Kinase involvement in these diverse pathways makes the superfamily of protein kinases a large and important class of enzymes in cellular signal transduction. The first tyrosine kinase to be identified was v-Src, which is responsible for the transformation of fibroblasts by the Rous sarcoma virus (RSV) [2]. The origin [3], regulation [1,4–6] and structure of v-Src [7–9], as well as of its cellular homolog c-Src [10–11], have been studied extensively and are well understood. The two kinases differ in several respects; many v-Src isolates contain a carboxy-terminal deletion which results in the loss of the inhibitory Tyr527. Another frequent mutation to the c-Src gene is found at position 338 where isoleucine is substituted for Thr338. The carboxy-terminal deletion or the Thr338→Ile mutation are individually sufficient, but not necessary, for transforming activity of v-Src. The latter mutation alone results in a v-Src gene which is only partially transforming [12].

The v-Src kinase is highly active. Over 50 proteins become phosphorylated (either directly or indirectly) by v-Src upon RSV infection of fibroblasts [13]. Although v-Src has been intensely studied using almost every biochemical and genetic tool available, we still do not know whether many of these 50 proteins are direct v-Src targets or are targets of intermediary kinases [14]. Identification of the specific substrates of all protein tyrosine kinases is made difficult by the large number of cellular kinases (it is estimated that 2% of the mammalian genome encodes protein kinases [15]), by the overlapping target specificity displayed by tyrosine kinases [1–11,13–16], and by the very low abundance of phosphotyrosine (only 0.03% of cellular phosphoaminoacids are phosphotyrosine [17]).

We previously reported a protein-engineering-based method for identifying the direct substrates of v-Src [18]. To differentiate the cellular substrates of v-Src from all other kinase substrates, we mutated the ATP-binding site of v-Src such that the engineered kinase uniquely

Table 1

Kinetic constants for phosphorylation of the IYGEFKKK peptide by wild-type and mutant v-Src kinases.

	GST–XD4 (wild-type v-Src)			GST–XD4 (I338A)			GST–XD4 (I338G)		
Nucleotide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,M^{-1}$)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,M^{-1}$)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,M^{-1}$)
ATP	2 ± 0.5	12 ± 3	1.6×10^5	1 ± 0.3	70 ± 10	1.4 ± 10^4	0.8 ± 0.3	80 ± 10	1×10^4
N^6-(cyclopentyl) ATP		> 2000 (K_i)		$(2.5 \pm 1) \times 10^{-2}$	40 ± 10	6.2 ± 10^2	0.1 ± 0.05	15 ± 3	6.7×10^3
N^6-(benzyl) ATP		> 2000 (K_i)		0.5 ± 0.2	20 ± 4	2.5×10^4	0.2 ± 0.1	5 ± 2	4.0×10^4

Kinetic constants were measured at low substrate conversion (< 5%) in triplicate and were determined by analysis of Lineweaver-Burk plots of the rate data. Reactions were performed in the same manner as in Figure 3 except for substitution of [γ-^{32}P] ATP by [γ-^{32}P] N^6-(cyclopentyl) ATP or [γ-^{32}P] N^6-(benzyl) ATP (both 5000 cpm/pmol) as indicated. K_i is the inhibition constant for the individual analog.

accepted a synthetic N^6-substituted ATP analog (A*TP; N^6-(cyclopentyl) ATP. The mutant v-Src was designed with four criteria in mind: it should be able to accept an ATP analog that is orthogonal (i.e., not a substrate for any wild-type protein kinase); it should use the A*TP with high catalytic efficiency; it should exhibit reduced catalytic efficiency with ATP, so that [γ-^{32}P] A*TP is the preferred substrate in the cellular milieu where ATP is present at high concentrations; and it should have the same substrate specificity as nonengineered ('wild type') v-Src. Initially, we identified two residues (Val323 and Ile338) in the v-Src catalytic domain which appeared to control specificity for N^6-substituted ATP analogs. Alanine mutagenesis of both residues (Val323→Ala, V323A and Ile338→Ala, I338A) in a v-Src fusion protein, GST–XD4 (V323A, I338A), produced a kinase capable of accepting an ATP analog, N^6-(cyclopentyl) ATP, which is not accepted by wild-type kinases. The engineered double-alanine mutant of v-Src in combination with N^6-(cyclopentyl) ATP satisfied three of our four design criteria, but only partially satisfied the requirement for preferential use of A*TP. The catalytic efficiency of the doubly mutated kinase with N^6-(cyclopentyl) ATP ($k_{cat}/K_M = 3.3 \times 10^3\ min^{-1}\,M^{-1}$ [18]) was comparable to the efficiency of the mutant with ATP ($k_{cat}/K_M = 5.3 \times 10^3\ min^{-1}\,M^{-1}$ [18]), but was significantly lower than the efficiency of the wild-type v-Src with ATP ($k_{cat}/K_M = 1.6 \times 10^5\ min^{-1}\,M^{-1}$; Table 1).

The 50-fold lower efficiency of mutant v-Src (V323A, I338A) with N^6-(cyclopentyl) ATP compared to the wild-type kinase could be considered a success in terms of engineering novel enzyme specificity. Our goal, however, was to design a mutant kinase capable of competing with wild-type kinases in a cellular context, and thus the mutant kinase is required to display close to wild-type catalytic efficiency with the unnatural triphosphate. If this design criterion is not adequately satisfied, the targets of mutant v-Src will be only minimally phosphorylated in the presence of active wild-type kinases and ATP. In fact, when we attempted to radiolabel direct substrates of v-Src (V323A, I338A) using the doubly mutated v-Src and [γ–^{32}P] N^6-(cyclopentyl) ATP, we observed suboptimal labeling of v-Src's substrates because of competition between wild-type kinases and the less active mutant kinase for N^6-(cyclopentyl) ATP (K.S., Y.L. and K.M.S., unpublished observations). To overcome this problem, we sought to re-engineer v-Src, as well as search for new analogs of ATP, in order to enhance the catalytic activity of the mutant v-Src with A*TPs.

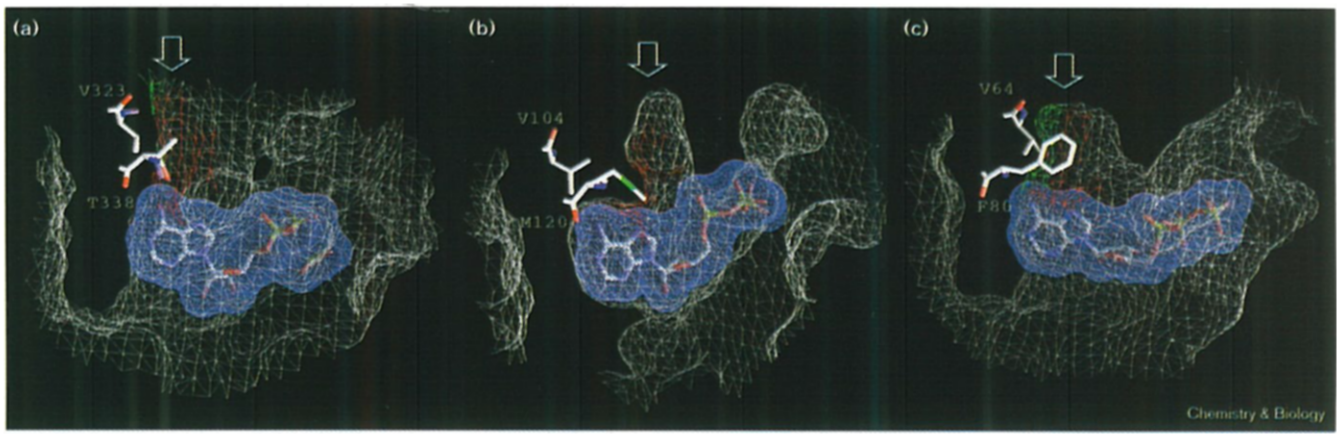
Here we report a site-directed mutagenesis study of residues in the v-Src active site which contact the N^6 amine of ATP. The mutant v-Src proteins were tested as catalysts of peptide and protein phosphorylation using a variety of synthetic ATPs (A*TPs). After identification of the 'best match' between a v-Src mutant and an A*TP, we examined the general applicability of our strategy by mutagenizing the Src-related tyrosine kinase Fyn, which shares 85% sequence identity with v-Src. We show that the mutational strategy can be extended to other Src family kinases and, by further sequence comparisons, to other kinase families as well.

Results and discussion

Ile338 controls specificity for N^6-substituted ATP analogs

We previously showed that ATP analogs with N^6 substituents larger than an isopropoxy group are not accepted by wild-type protein kinases [18]. At that time there were no crystal structures of tyrosine kinases in an active conformation [19–21], so it was not easy to determine the residue(s) responsible for limiting the steric bulk of N^6 substituents on ATP. Inspection of the crystal structures of the serine/threonine kinases (cAMP-dependent kinase, PKA [21] and the cyclin-dependent kinase CDK2 [22]) in the region around the N^6 amine of bound ATP revealed two amino acid sidechains within 5 Å of the N^6 amino group of ATP. Mutation of the corresponding residues in v-Src to the less sterically demanding residue alanine afforded a protein kinase which utilized ATP analogs with bulky N^6 substituents, in agreement with our predictions from homology modeling (shown in [18]).

Figure 1



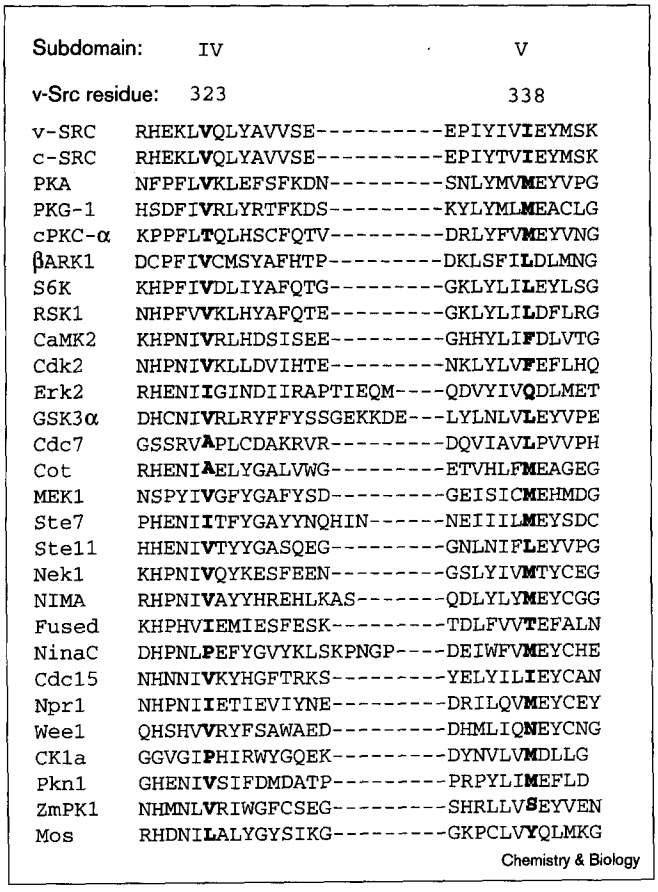
Surface representation of the ATP-binding pocket in three distantly related protein kinases: the Src family member Hck, PKA, and CDK2. The solvent-accessible surface of each kinase within 7 Å of ATP is shown in white mesh, and the surface of ATP is shown in blue mesh. **(a)** Wild-type Hck + AMP–PNP. The portion of Hck's solvent-accessible surface area formed by Thr338 (corresponding to Ile338 in v-Src) is shown in red mesh and the portion of the surface formed by Val323 is shown in green mesh. **(b)** Wild-type PKA + ATP. The portion of PKA's solvent-accessible surface area formed by Met120 (corresponding to Ile338 in v-Src) is shown in red mesh and the portion of the surface formed by Val104 is shown in green mesh. **(c)** Wild-type CDK2 + ATP. The portion of CDK2's solvent-accessible surface area formed by Phe80 (corresponding to Ile338 in v-Src) is shown in red mesh and the portion of the surface formed by Val64 is shown in green mesh. ATP, and the residues corresponding to Ile338 and Val323 in v-Src in each kinase (a–c) are shown in stick representation with the following atom coloring: O, red; N, blue; C, white; P, yellow. These figures were created using the molecular modelling program GRASP [41].

Recently, structures of three Src-family kinases (c-Src [11], Hck [23], and Lck [24]) have made possible a more detailed analysis of the similarities between the two families of kinases (serine/threonine and tyrosine kinases) with respect to the pocket around the N^6 amine of ATP. The ATP-binding pockets of the three distantly related kinases are shown in Figure 1 in surface representation. We analyzed the kinase active sites, looking for a conserved structural feature which could explain the inability of wild-type kinases to accept N^6-substituted ATP analogs. It was immediately apparent that the shape complementarity between the kinase active site and the N^1 and N^6 positions of the ATP purine ring is well conserved in all three kinases. In particular, there is little unoccupied space adjacent to the N^6 amine of ATP in any of the available kinase crystal structures.

We next turned to sequence alignments of all protein kinases to identify conserved residues which would be responsible for forming the complementary interactions with the N^6 position of ATP in all kinases. Surprisingly, analysis of kinase sequence alignments indicated that the two residues which make close contact with the N^6 amine of ATP (Val323, Ile338 in v-Src) are not completely conserved in all kinases, as the three-dimensional structures might have suggested (Figure 2). Although the residue that corresponds to Val323 in v-Src is most often a β-branched residue (a residue which contains two non-hydrogen atoms at the β carbon), it can also be a small unbranched residue, such as alanine (see Figure 2, red letters). The fact that several kinases contain alanine at this position suggested to us that this residue might not be responsible for limiting the steric bulk of N^6 substituents on ATP. We also analyzed the crystal structures of Hck, PKA, and CDK2 in terms of the importance of residues that correspond to Val323 of v-Src in forming the pocket around the N^6 position of ATP. The surface formed by Val323 in Hck is colored in green mesh (Figure 1a). Only a small patch of green is visible because Val323 does not significantly contribute to forming the N^6-binding pocket of Hck. Similarly, the residue that corresponds to Val323 in PKA (Val104, green surface in Figure 1b) does not contribute to the ATP-binding pocket in PKA. Interestingly, by contrast, the corresponding residue in CDK2, Val64 (green mesh in Figure 1c), does contribute significantly to the N^6-binding pocket in this kinase.

The other residue in the vicinity of the N^6 group that corresponds to Thr338 in c-Src (see Figure 2; interestingly, this residue is mutated to isoleucine in some v-Src isolates, see below) is quite structurally conserved across the kinase family in that no kinases contain small unbranched alanine or glycine residues at this position (Figure 2). In fact, the smallest residue in any kinase at this position is serine (Figure 2, purple S). Structural comparison of the contribution of this residue to the N^6-binding pocket of ATP in the three kinases analyzed (shown in red mesh, Figure 1) confirms that a significant portion of the ATP-binding pocket in the region around the N^6 amine is formed by this single residue. On the

Figure 2



Sequence alignment of subdomains IV and V of representative protein kinases from the protein kinase superfamily of Hanks and Quinn [42]. Residues predicted to be within 5 Å of the N^6 amine of bound ATP (Val323 and Ile338, v-Src numbering) are shown in bold. At least two protein kinases, Cot and Cdc7, naturally contain residues with small sidechains – alanine in both cases, highlighted in red – at the position that corresponds to position 323 in v-Src. The smallest naturally occurring residue at position 338 in the databank is serine (shown in purple, in ZmPK1).

basis of these structural and sequence alignment analyses, we propose that the single bulky residue at the position corresponding to Ile338 in v-Src in most if not all kinases is primarily responsible for restricting the ability of wild-type kinases to accept N^{6-} substituted ATP analogs.

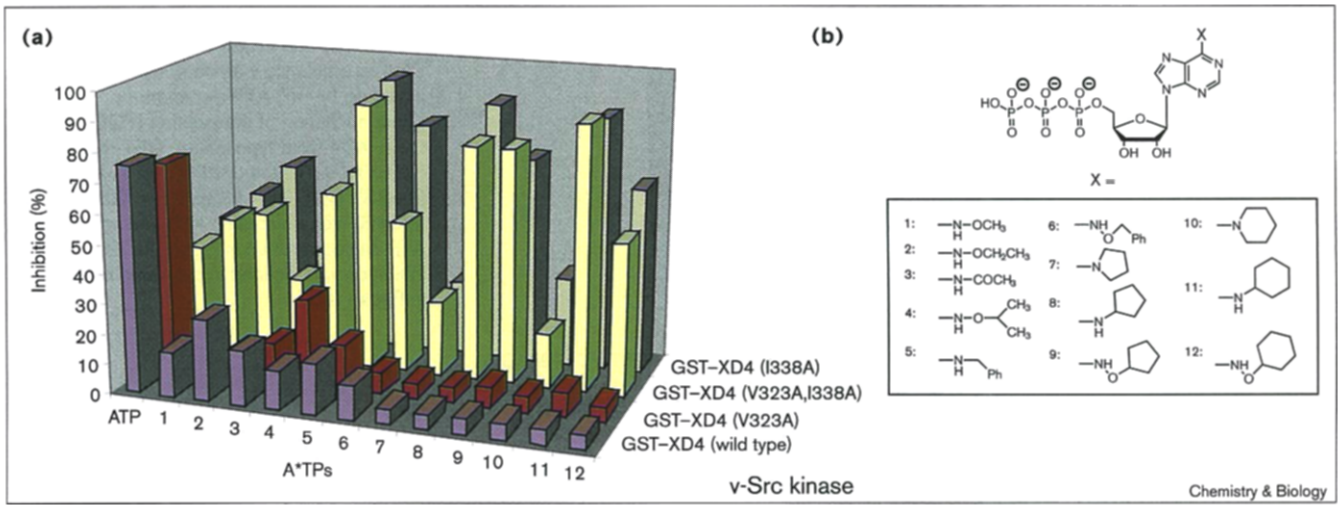
Although structural and sequence analyses provide good starting points for designing novel enzyme properties, an optimal design usually requires an iterative process of mutation, kinetic analysis, and further structural analysis [25]. In order to explore the pocket dimensions and geometries of our mutant v-Src (V323A, I338A) more fully, we asked whether mutation of residue 338 and not residue 323 was necessary and sufficient for v-Src to accept bulky ATP analogs. We used site-directed mutagenesis to construct single mutants of v-Src, GST–XD4 (V323A), and GST–XD4 (I338A) and evaluated their binding affinity for the A*TPs shown in Figure 3b. The A*TPs were tested as inhibitors of [γ-^{32}P] ATP-dependent phosphorylation of a peptide substrate (IYGEFKKK; using single letter amino-acid code) [16]. The single mutant GST–XD4 (V323A) displays almost the same inhibition pattern as wild-type v-Src (GST–XD4, Figure 3), suggesting that mutation of this residue to alanine is not important in enlarging the ATP-binding pocket near the N^6 amine of ATP. In contrast, phosphorylation by the single mutant GST–XD4 (I338A) is inhibited by bulky A*TPs (Figure 3), suggesting that residue 338 is what controls binding of N^6-substituted A*TPs to wild-type v-Src.

We anticipated that if a single substitution in v-Src (I338A) was sufficient to confer A*TP binding, disruption of the kinase's active site might be less than in the double mutant [18], and so the I338A kinase might display higher catalytic efficiency than the double mutant (V323A, I338A). The single mutation to v-Src (I338A) however, displays the same lower catalytic efficiency with ATP as a phosphodonor as is found with the V323A, I338A double mutant (Figure 4, lanes 2,4). Interestingly, the single mutation to v-Src which does not confer A*TP binding (V323A) shows a catalytic efficiency similar to that of the wild-type form of the kinase with ATP (Figure 4, lanes 1,3). Unfortunately, the V323A mutant does not accept N^6-(cyclopentyl) ATP as a phosphodonor for autophosphorylation (Figure 4, lane 7), so it does not satisfy all of our design requirements. Although we have identified the minimal perturbation to the v-Src structure which is sufficient to confer A*TP binding, the mutation results in a twofold loss of catalytic activity (k_{cat}) when ATP is a substrate. The space-creating mutation I338A may be removing an important interaction with the natural substrate ATP in the transition state.

Plasticity at position 338

Our alanine scanning mutagenesis study of residues with sidechains in the 5 Å sphere of the N^6 group in ATP suggested Ile338 in v-Src controls A*TP specificity. We carried out a more focused mutagenesis study at position 338, in order to search for a mutant that displays efficient A*TP analog binding and high catalytic efficiency. We mutated Ile338 to valine, serine, or cysteine to determine whether residues larger than alanine but smaller than the isoleucine or threonine found in the wild-type kinases would display the desired catalytic activity and A*TP binding. These mutants display poor binding affinity for large N^6-substituted analogs, and their specificity for other analogs is essentially like that of the wild-type kinase (Figure 5). This analysis suggests that alanine is the largest amino acid that can confer A*TP binding on v-Src. Moreover, no known kinases (http://www.sdsc.edu/kinases/pkr/pk_catalytic/) naturally contain an alanine at the position corresponding to Ile338 in v-Src. This sequence conservation might be responsible for our

Figure 3

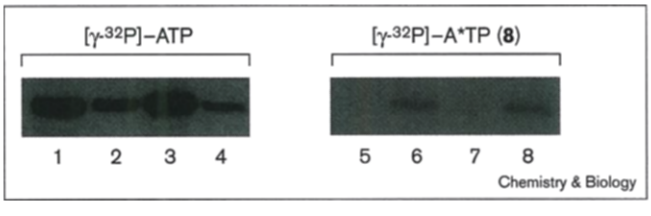


(a) Evaluation of the binding efficiency of A*TPs to wild-type and Val323 and Ile338 mutant v-Src kinases. Binding efficiency was determined by inhibiting [γ-^{32}P] ATP-dependent phosphorylation of the peptide IYGEFKKK by GST–XD4 (wild-type v-Src), GST–XD4 (V323A), GST–XD4 (V323A, I338A) and GST–XD4 (I338A) with unlabelled ATP and ATP analogs **1–12**. Percentage inhibition is calculated as $(1 - v_i/v_0)$ where v_i is the disintegration rate (cpm) in the presence of 100 μM of the indicated triphosphate and 10 μM [γ-^{32}P] ATP (1000 cpm/pmol) and v_0 is the disintegration rate (cpm) in the presence of 10 μM [γ-^{32}P] ATP (1000 cpm/pmol) alone. **(b)** Structures of ATP analogs (A*TPs; **1–12**) used in this experiment.

observation that no wild-type kinases are able to accept N^6-(cyclopentyl) ATP as a substrate [18].

The only remaining substitution at position 338 that is predicted to allow catalysis with A*TP is glycine, because it is smaller than alanine. Indeed, GST–XD4 (I338G) shows the same inhibition pattern as that of GST–XD4 (I338A) with a slightly enhanced binding affinity for the analogs containing larger N^6 groups (Figure 5). It therefore appears that residues larger than alanine at position 338 preclude binding of A*TPs whereas alanine and glycine at this position confer on v-Src the ability to bind A*TPs that have bulky N^6 substituents.

Figure 4

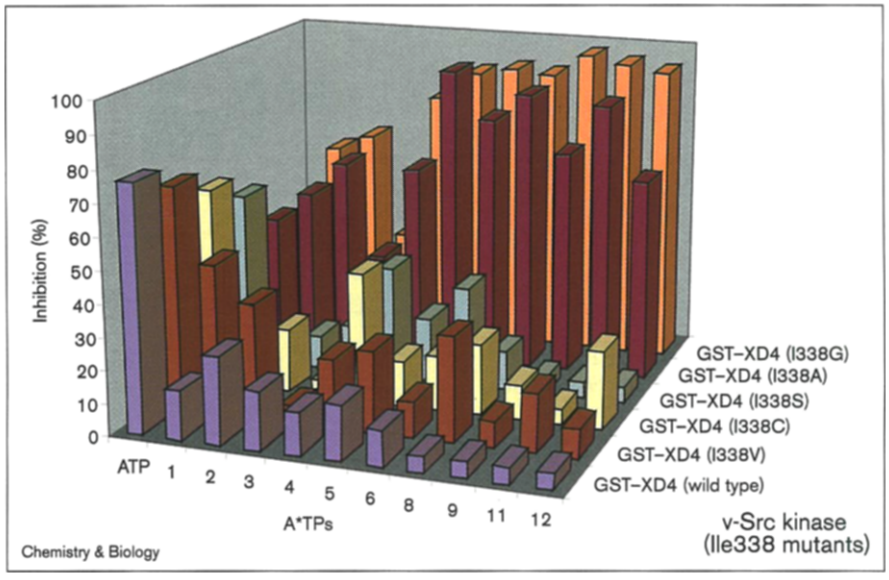


Autoradiograms showing [γ-^{32}P]ATP-dependent autophosphorylation of GST–XD4 (wild-type v-Src), lane 1; GST–XD4 (V323A, I338A), lane 2; GST–XD4 (V323A), lane 3; GST–XD4 (I338A) lane 4; and [γ-^{32}P] N^6-(cyclopentyl) ATP-dependent phosphorylation of GST–XD4 (wild-type v-Src), lane 5; GST–XD4 (V323A, I338A), lane 6; GST–XD4 (V323A), lane 7; GST–XD4 (I338A), lane 8. The autophosphorylation reaction was carried out as described in the Materials and methods section.

Structural context of residue 338

The crystal structure of c-Src shows that Thr338 is at the back of the nucleotide-binding pocket in the interdomain hinge which links the catalytic amino- and carboxy-terminal domains [11]. Mutation of v-Src Ile338 to glycine is predicted to have two effects on the enzyme — enlarge the ATP-binding pocket, and lend more flexibility to interdomain hinge [26]. We asked which of these two effects is more important for conferring A*TP specificity on v-Src; we attempted to increase the flexibility of the interdomain hinge without enlarging the ATP-binding pocket, and asked whether this allowed v-Src to accept A*TPs. We focused on residue Glu339 in the hinge, because the carboxylate sidechain of Glu339 is oriented away from the interior of the ATP-binding site in all existing kinase crystal structures; thus its mutation to alanine or glycine was not expected to affect the N^6 pocket dimensions. Because it is the residue adjacent to Ile338 in the interdomain hinge, addition of a glycine residue was expected to enhance the flexibility of the hinge to a similar extent as in the I338G mutant. We found that the GST–XD4 (E339G) mutant is inhibited to a certain extent by N^6-substituted ATP analogs, compared with wild-type v-Src, but not nearly to the extent found in GST–XD4 (I339G). In Figure 6, the I338G mutant shows 97% inhibition by N^6-(cyclopentyloxy) ATP whereas the E339G mutant shows only 42% inhibition by the same A*TP. This analysis demonstrated that interdomain flexibility has an effect on A*TP binding, but relief of steric congestion in the ATP-binding pocket adjacent to the N^6

Figure 5



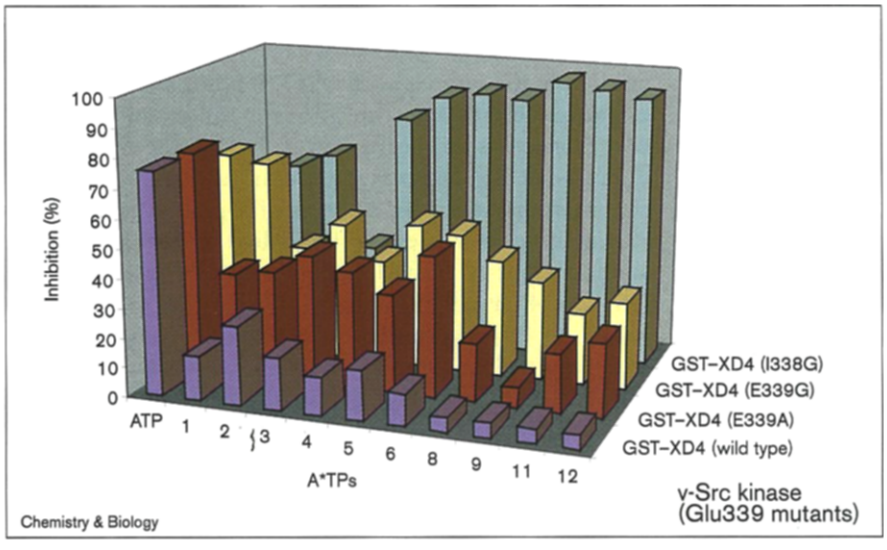
Evaluation of the binding efficiency of A*TPs to wild-type and Ile338 mutant v-Src kinases. Binding efficiency was determined by inhibiting [γ-^{32}P] ATP-dependent phosphorylation of the peptide IYGEFKKK by GST–XD4 (wild-type v-Src), GST–XD4 (I338V), GST–XD4 (I338C), GST–XD4 (I338S), GST–XD4 (I338A) and GST–XD4 (I338G) with unlabelled ATP and some of the ATP analogs shown in Figure 3b. Percentage inhibition was defined in the same manner as in Figure 3a.

amine is the most critical feature for engineering unnatural ATP analog specificity.

Determining the optimal ATP analog for I338A or I338G mutants of v-Src

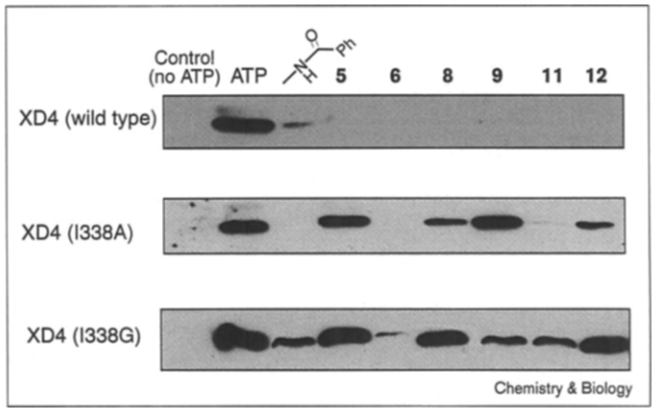
Although N^6-(cyclopentyl) ATP was identified as the optimal substrate for the first-generation double-alanine mutant (V323A, I338A) [18], it was not unreasonable to expect that each new kinase mutant might exhibit subtle differences in the ability to catalyze phosphorylation with other A*TPs. Because our analysis of A*TP binding to v-Src mutants yielded two new v-Src mutants, I338A and I338G, we decided to probe these two new kinases with all the available A*TPs (Figure 3b) to find the best match of unnatural substrate (A*TP) and mutant enzyme. Using [γ-^{32}P] ATP as the phosphodonor and an optimal peptide substrate (IYGEFKKK), the binding affinity of v-Src mutants for A*TPs can be measured using an inhibition assay. The disadvantage of such an assay is that analogs which have tight binding constants are not necessarily good substrates. One solution to this problem is to synthesize [γ-^{32}P]-labeled A*TPs and to test them in a peptide phosphorylation assay; this is not very appealing, however, as it involves a great deal of radioactive synthesis. We chose to use a nonradioactive assay which could directly test nonradiolabeled A*TPs as substrates of mutant

Figure 6



Evaluation of the binding efficiency of A*TPs to wild-type and Ile338 and Glu339 mutant v-Src kinases. Binding efficiency was determined by inhibiting [γ-^{32}P] ATP-dependent phosphorylation of the peptide IYGEFKKK by GST–XD4 (wild-type v-Src), GST–XD4 (I339A), GST–XD4 (E339G) and GST–XD4 (E338G) with unlabelled ATP and and some of the ATP analogs shown in Figure 3b. Percentage inhibition was determined in the same manner as in Figure 3a.

Figure 7



Anti-phosphotyrosine immunoblot of GFP–IYGEF phosphorylation with ATP and various ATP analogs by GST–XD4 (wild-type v-Src), GST–XD4 (I338A) and GST–XD4 (I338G). Lane 1, control, no ATP; lane 2, ATP; lane 3, N^6-(benzoyl) ATP (the ATP N6 substitution is shown above the lane); lane 4, N^6-(benzyl) ATP (**5**); lane 5, N^6-(benzyloxy) ATP (**6**); lane 6, N^6-(cyclopentyl) ATP (**8**); lane 7, N^6-(cyclopentyloxy) ATP (**9**); lane 8, N^6-(cyclohexyl) ATP (**11**); lane 9, N^6-(cyclohexyloxy) ATP (**12**). Reaction conditions are described in the Materials and methods section.

kinases. The best existing nonradioactive assay is a coupled ADP-formation assay that uses ADP-requiring enzymes, but this cannot be extended to our A*DP products [27]. We therefore developed a new assay that utilizes a highly specific anti-phosphotyrosine antibody to detect phosphorylation of a specific tyrosine on an engineered substrate (green fluorescent protein carboxy-terminally tagged with an optimal Src substrate EIYGEF, designated 'GFP-IYGEF'; FY., Y.L., SD. Bixby, J.D. Friedman and K.M.S., unpublished observations).

The anti-phosphotyrosine immunoblot of GFP–IYGEF phosphorylation by v-Src with orthogonal ATP analogs (A*TP analogs not accepted by wild-type kinases) is shown in Figure 7. The best substrate for both the I338A and I338G mutants is N^6-(benzyl) ATP, as measured by the intensity of the anti-phosphotyrosine immunoblot signal (Figure 7, lane 7). The inhibition assay (Figure 5) shows that the I338A and I338G v-Src mutants display similar binding affinity with analogs **5**, **6**, **8**, **9**, **11**, and **12** (Figure 5), yet the catalytic activity assay demonstrates that these two mutants use these ATP analogs with quite different catalytic efficiencies.

The GST–XD4 (I338G) mutant shows the highest inhibition with **9**, N^6-(cyclopentyloxy) ATP, (Figure 5) but this analog is not as good a substrate as either N^6-(benzyl) ATP or N^6-(cyclopentyl) ATP (Figure 7, lanes 4 and 6, respectively, versus lane 7). The N^6-(cyclopentyloxy) ATP, is in fact, a potent inhibitor of the I338G v-Src mutant (IC_{50} = 0.05 μM, data not shown). It is a poor inhibitor of the I338G mutant (IC_{50} = 80 μM, data not shown). In terms of catalytic efficiency, however, the I338A mutant uses N^6-(cyclopentyloxy) ATP with a catalytic efficiency higher than the I338G mutant. In our search for unnatural substrates of engineered enzymes, therefore, we have actually identified several ATP analogs which are highly selective inhibitors of the various mutant kinases [28].

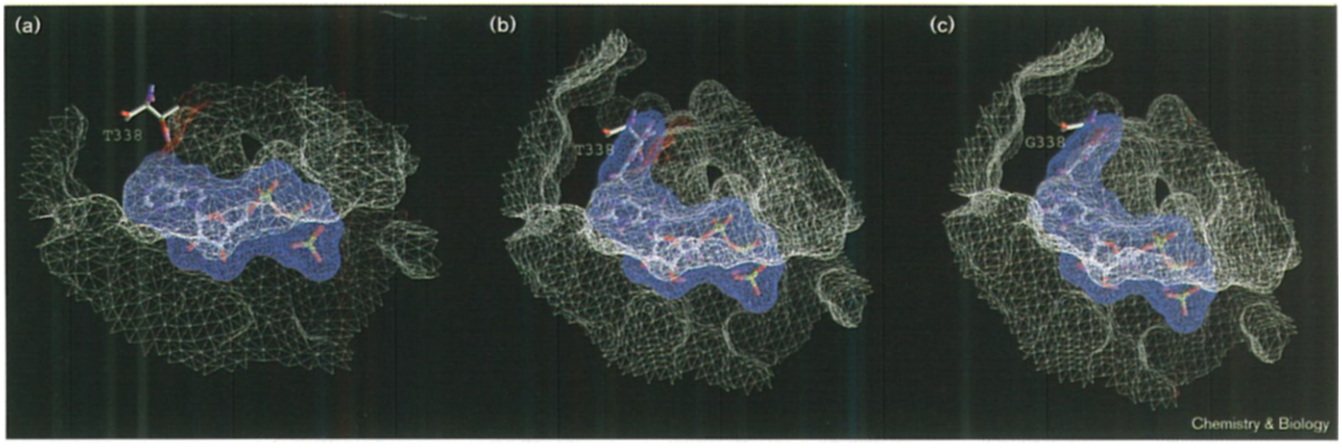
In order to determine the catalytic rate constants for mutant v-Src phosphorylation we synthesized [γ-^{32}P] N^6-(benzyl) ATP, the best analog identified in the screen of our 12 A*TPs. The wild-type kinase GST–XD4 did not phosphorylate the IYGEFKKK peptide with [γ-^{32}P] N^6-(benzyl) ATP, confirming our previous observation that this nucleotide analog is not a wild-type substrate. GST–XD4 (I338A) displays a k_{cat} of 0.5 min^{-1} with N^6-(benzyl) ATP, 20 times higher than with N^6-(cyclopentyl) ATP (0.025 min^{-1}; Table 1). The k_{cat}/K_m of GST–XD4 (I338G) with N^6-(benzyl) ATP (4.0×10^4 min^{-1} M^{-1}) is fourfold higher than with ATP (1.0×10^4 min^{-1} M^{-1}) and only fourfold less than wild-type kinase GST–XD4 with ATP (1.6×10^5 min^{-1} M^{-1}). A 20-fold improvement of the catalytic efficiency of our mutant v-Src demonstrates the advantage of iterative mutagenesis and analog screening. Even more significant than the absolute increase in catalytic activity of the mutated kinase is the switch in the preferred substrate, from ATP to an A*TP. Our first generation v-Src mutant (V323A, I338A) preferentially utilizes ATP over N^6-(cyclopentyl) ATP (k_{cat}:K_m ratio 6:1), whereas I338G v-Src preferentially uses N^6-(benzyl) ATP over ATP (k_{cat}:K_m ratio 4:1).

The key finding from our efforts to engineer v-Src to efficiently catalyze phosphotransfer reactions is that residue 338, a bulky residue in all known kinases (see Figure 1), limits the ability of v-Src to bind N^6-substituted A*TPs (Figure 8a,b). Mutation of this residue to alanine or glycine confers on v-Src the ability to accept N^6-substituted A*TPs (Figure 8c). A screen of 12 candidate A*TPs with various substituents at the N^6 position of ATP, identified N^6-(benzyl) ATP as the best A*TP substrate. As protein kinases share a common protein fold, we reasoned that mutation of residues corresponding to 338 (in v-Src) might confer on other kinases the ability to accept N^6-(benzyl) ATP (or another A*TP from our panel).

Engineering Fyn, a Src family kinase, to accept an A*TP

The Src family of tyrosine kinases contains nine members: Src, Lck, Fyn, Lyn, Hck, Yes, Fgr, Blk and Yrk [5], which are involved in processes such as lymphocyte development, platelet activation, and mast cell degranulation [29–31]. The amino acid at the position corresponding to 338 in v-Src is either threonine or isoleucine

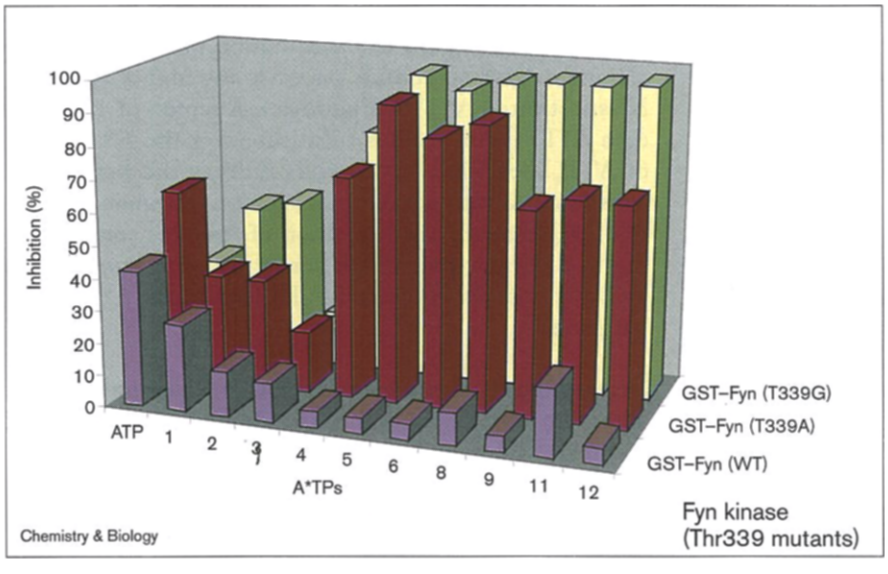
Figure 8



Surface representation of the ATP-binding pocket in the Src family tyrosine kinase Hck and the proposed effect of enlarging the ATP-binding pocket to accommodate N^6-(benzyl) ATP. **(a)** Wild-type Hck + AMP–PNP. The solvent-accessible surface of Hck within 7 Å of ATP is shown in white mesh, the surface of ATP is shown in blue mesh. The portion of Hck's solvent-accessible surface area formed by Thr338 (corresponding to Ile338 in v-Src) is shown in red mesh. Atoms are colored the same as in Figure 1. Complementary packing of ATP into the wild-type Hck active site indicates the proximity of the N^6 amine of ATP to residue 338. **(b)** Wild-type Hck + N^6-(benzyl) AMP–PNP. Model of the unfavorable packing of N^6-(benzyl) ATP into the wild-type Hck active site, showing the steric clash between the benzyl ring of N^6 (benzyl)ATP and the sidechain of Thr338. **(c)** Mutant Hck (T338G) + N^6-(benzyl) AMP–PNP. Model of the improved fit of N^6-(benzyl) ATP into the enlarged T338G mutant Hck ATP-binding pocket. The conformation of the N^6 benzyl ring was chosen by manually checking multiple rotamers of the N–C and C–Ph bonds in N^6-(benzyl) ATP for steric clashes with the residues in the Hck active site using the program InsightII. The rotamer with the least steric clashes determined in this manner was then imported into the program GRASP (A. Nichols and B. Honig, Columbia University) to produce the surface maps. These figures were created using GRASP [41]. (We show Hck instead of Src because the structure of Src complexed to ATP is not available.)

in all oncogenic or proto-oncogenic Src-family kinases. Mutation of Thr338 in c-Src to isoleucine is known to be sufficient for partial transformation of chicken embryo fibroblasts [12], as this single mutation is known to increase the specific activity of the c-Src kinase. Several v-Src isolates contain threonine at position 338, and thus isoleucine is not necessary to cause transformation of fibroblasts by v-Src [32]. The activating nature of the threonine to isoleucine mutation at this position could be the result of loss of a hydrogen bond between the

Figure 9



Evaluation of the binding efficiency of A*TPs to wild-type and mutant Fyn kinases. Binding efficiency was determined by inhibiting of [γ-^{32}P] ATP-dependent phosphorylation of the specific peptide IYGEFKKK by GST–Fyn, GST–Fyn (T339A) and GST–Fyn(T339G) with unlabelled ATP and A*TPs. Percentage inhibition was defined in the same manner as in Figure 3.

Table 2

Kinetic constants for phosphorylation of the IYGEFKKK peptide by wild-type and mutant Fyn kinases.

	GST–Fyn (wild type)			GST–Fyn (T339A)			GST–Fyn (T339G)		
Nucleotide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,M^{-1}$)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,M^{-1}$)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,M^{-1}$)
ATP	2.5 ± 0.5	70 ± 10	3.6×10^4	0.8 ± 0.2	90 ± 10	9.0 ± 10^3	0.5 ± 0.2	100 ± 10	5.0×10^3
N^6-(benzyl) ATP		> 2000 (K_i)		0.5 ± 0.2	25 ± 5	2.0×10^4	0.3 ± 0.2	7 ± 2	7.5×10^4

The kinetic constants were determined in the same manner as in Table 1.

hydroxyl group in Thr338 and bound ATP [11]. We chose Fyn as a test of the generality of our engineering method for several reasons: it is closely related to Src (sharing 85% sequence identity); it is a nontransforming kinase and thus has different regulatory domains which are not present in v-Src; and it is a critical kinase in multiple signaling pathways including the T cell receptor pathway, neuronal signaling, and the control of cell growth. The ability to identify Fyn's substrates in all relevant cellular contexts is an important goal. The first step in using our protein-based approach was to engineer Fyn to accept an A*TP.

Full-length mouse Fyn tyrosine kinase was expressed as a GST-fusion protein in bacteria. It has a k_{cat} of 2.5 min^{-1} using the peptide substrate IYGEFKKK and ATP. Using our mutagenesis of v-Src as a guide, we mutated position 339 in Fyn from threonine to alanine or glycine; this allowed us to test how generally applicable our method is to kinases which contain a different residue than that found in v-Src.

Both GST–Fyn (T339A) and GST–Fyn (T339G) show excellent inhibition by the same set of A*TPs which inhibit the corresponding alanine and glycine mutants in v-Src (Figure 9). In fact, both Fyn mutants catalyze peptide phosphorylation with N^6-(benzyl) ATP efficiently (Table 2). The k_{cat}/K_m of both mutants with N^6-(benzyl) ATP (2.0×10^4, 7.5×10^4 $min^{-1}\,M^{-1}$ for the alanine and glycine mutants, respectively) is higher than with ATP (9.0×10^3, 5.0×10^3 $min^{-1}\,M^{-1}$ for the alanine and glycine mutants, respectively) and comparable with the wild-type Fyn with ATP (3.6×10^4 $min^{-1}\,M^{-1}$). The relative efficiencies of the Fyn T339A, T339G mutants with N^6-(benzyl) ATP versus ATP parallel those found with v-Src mutants, further confirming the functional and structural conservation of the active site within the Src family of kinases. In fact, mutation of position 339 to alanine or glycine had a less detrimental effect in the overall catalytic efficiency when compared to wild-type Fyn than was found in the case of v-Src. This analysis suggests the Fyn mutant with A*TP is a nearly perfect mimic of wild-type Fyn with ATP, in terms of catalytic efficiency.

Significance

Protein kinases play a central role in signal transduction. Nearly half the oncogenes that have been identified are protein kinases [33]. Identification of the *in vivo* substrates of protein kinases will provide a more detailed understanding of signaling cascades and also uncover important targets for rational drug design. Tremendous redundancy and overlapping substrate specificities among protein kinases has made it difficult to dissect the individual signaling pathways by scanning sequences for a kinase's unique substrate motifs. We have developed a protein engineering method to uniquely tag the direct substrates of a protein kinase of interest in the presence of many other cellular kinases. A detailed mutagenesis study of the prototypical tyrosine kinase, v-Src, is described here. We have created mutants of v-Src which can catalyze phosphorylation reactions with ATP analogs (A*TPs) that are not accepted by wild-type kinases, including v-Src itself. Specifically, we identified a single mutation in the kinase domain which controls specificity for N^6-substituted ATP analogs; we also identified an ATP analog which is an efficient substrate for the mutant kinase. The mutant kinase–ATP analog pair displays catalytic efficiency comparable to that of the wild-type kinase with the natural substrate ATP. With this v-Src mutant and ATP analog, we can begin to identify the direct substrates of v-Src. Furthermore, the successful engineering of Fyn, another kinase for the Src family, demonstrates that this approach can be extended to other, non-oncogenic protein kinases. The new method for identifying direct protein kinase substrates will make it possible to dissect individual signaling pathways.

Materials and methods

Synthesis of ATP analogs

ATP analogs **1**-**12** were synthesized as described previously [18].

Peptide synthesis

The tyrosine kinase substrate peptide, IYGEFKKK, was synthesized on an Applied Biosystems AB431A automatic solid-phase peptide synthesizer using a standard Fmoc peptide synthesis protocol [34,35] and WANG resin. Upon completion of peptide synthesis, the sidechain protected (tBu for tyrosine and glutamic acid, Boc for lysine) peptide was cleaved from the resin using Reagent K [36]. Isolation via ether precipitation yielded peptides of sufficient purity (> 98% as determined via

reverse-phase high performance liquid chromatography [HPLC] and peptide sequencing) for the kinase reaction.

Site-directed mutagenesis, protein expression and protein purification

Overlap extension polymerase chain reaction (PCR) was used to make GST–XD4 (V323A) and GST–XD4 (I338A/G) [37]. Pfu polymerase (from Stragene) was used according to the manufacturer's protocol. Eight synthetic oligonucleotides were used to generate the GST–XD4 v-Src and GST–Fyn mutant kinases: primer 1 (5′-TTT*GGATCC*ATG-GGGAGTAGCAAGAGCAAG), primer 2 (5′-TTT*GAATTC*CTACTCA-GCGACCTCCAACAC), primer 3 (5′-TGAGAAGCTGG**C**TCAACTGT-ACGCAG), primer 4 (5′-CTGCGTACAGTTGA**G**CCAGCTTCTCA), primer 5 (5′-CTACATCGTC**GC**TGAGTACATGAG), primer 6 (5′-CT-CATGTACTCA**GC**GACGATGTAG), primer 7 (5′-CTACATCGTC**GGG**-GAGTACATGAG), primer 8 (5′-CTCATGTACTC**CCC**GACGATGTAG). Note that italics indicate restriction enzyme sites and bold indicates where mutations were induced. Primer 1 includes a *Bam*H1 site and primer 2 has an *Eco*R1 site. Primers 3 and 4 contain the I338G mutation. Primers 5 and 6 contain the I338A mutation. Primers 7 and 8 contain the I338G mutation. The XD4 gene from YEp51–XD4 plasmid was amplified using primers 1 and primer 2. The PCR product was digested with *Bam*H1 and *Eco*R1 and ligated into *Bam*H1 and *Eco*R1-digested pGEX–KT and then transformed into the *E. coli* strain DH5α. GST–XD4 (V323A) was made using primers 1, 2, 3 and 4 with the GST–XD4 (truncated v-Src) plasmid as the template. In the first PCR, primers 1 and 4 were used to produce one fragment and primers 2 and 4 were used to produce another fragment. In the second round of PCR, these two fragments were annealed and extended to form the full length GST–XD4 (V323A) gene. The PCR products were digested with *Bam*H1 and *Eco*R1 and ligated into a similarly digested pGEX–KT. The resultant plasmid was transformed into the *E. coli* strain DH5α. GST–XD4 (I338A) and GST–XD4 (I338G) were made in the same way except for the use of the corresponding internal primers (#5 and #6 for I338A, #7 and #8 for I338G). GST-Fyn (T339A) and GST-Fyn (T339G) were constructed using wild-type Fyn (murine Fyn in pSV7c, from Mike Cole, Princeton University) as the template. The primers corresponding to primers 1–6 used for GST–XD4 mutations are: primer 1′ (5′-TTTGGATCCATGGGCTGTGTGCAATGTAAG), primer 2′ (5′-TTTGAATTCTCACAGGTTTTCAACGGGCTG), primer 3′ (5′-CATTTACATCGTCG**CGG**AGTACATGAG), primer 4′ (5′-CTCAT-GTACT**CCG**CGACGATGTAAATG), primer 5′ (5′-CATTTACATCGT-CG**GGG**AGTACATGAG), primer 6′ (5′-CTCATGTACT**CCC**CGACG-ATGTAAATG). All mutants were sequenced over the entire coding region (Oligonucleotide sequencing and synthesis facility, Princeton University). Expression of kinases was carried out in DH5α as described by Xu *et al.* [38] with the exception that the cells were stored at 4°C overnight before centrifugation and lysis by French press (overnight storage is essential for producing highly active kinases).

In vitro *kinase peptide assay and autophosphorylation*

Assays of phosphorylation of the peptide substrate (IYGEFKKK) were carried out in triplicate at 22°C in a final volume of 30 µl buffered at pH 8.0 containing 50 mM Tris, 10 mM $MgCl_2$, 1.6 mM glutathione, 1 mg/ml BSA, 0.1 mM peptide with 100 nM of kinase, and 10 µM [γ-^{32}P]ATP (1000 cpm/pmol; DuPont/NEN). For inhibition studies, 100 µM unlabelled ATP or A*TPs (**1**–**12**) was added prior to the addition of the enzyme. After 30 min, reactions were quenched by spotting 25 µl of the reaction volume onto p81 phosphocellulose disks (Whatman) and immersing in 250 ml of 10% acetic acid for > 30 min, followed by washing in 0.5% phosphoric acid and scintillation counting according to standard methods [39]. Similar assays were used to measure k_{cat} and K_M values for the GST–kinases. Kinase autophosphorylation reactions were carried out in the presence of 50 µM [γ-^{32}P] ATP (5000 cpm/pmol) or [γ-^{32}P] N^6-(cyclopentyl) ATP (5000 cpm/pmol) at 22°C for 1 h in a final volume of 30 µl at pH 8.0, 50 mM Tris, 10 mM $MgCl_2$ and 10 pmol of kinase. After adding 6 µl 6X Laemmli gel loading buffer, proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS–PAGE. The gel was soaked in 10% acetic acid/10% isopropanol for 1 h, after which it was dried and exposed to film.

GFP–IYGEF Western blot assay

The phosphorylatable sequence IYGEF was added to the carboxyl terminus of GFP [40] and expressed in DH5α cells as 6-His-GFP–IYGEF (G.Y., Y.L., S.D. Bixby, J.D. Friedman and K.M.S., unpublished observations). Assays of GST–XD4 (I338A) and GST–XD4 (I338G) phosphorylation of GFP–IYGEF were carried out at 22°C in a final volume of 30 µl buffered at pH 8.0 containing 50 mM Tris, 10 mM $MgCl_2$, 20 µg/ml GFP–IYGEF and 50 µM ATP or A*TPs. After 30 minutes, the reactions were quenched by adding 6µl 6X Laemmli gel loading buffer. Proteins were separated using 12% SDS–PAGE. The gel was transferred to nitrocellulose paper (Schleicher & Schuell). The blot was probed with the anti-phosphotyrosine antibody 4G10, and the bound antibody was detected via enhanced chemiluminescence after treatment with goat anti-mouse antibody (VWR Scientific 7101332).

Acknowledgements

This work was supported by a National Science Foundation Early Career Development Award (MCB-9506929) and by the National Institutes of Health (1R011CA70331-01). K.M.S. is a Pew Scholar in the Biomedical Sciences. The YE p51–XD4 plasmid was a gift from Brent Cochran at Tuffs Medical School. We thank Mike Cole for plasmid pSV7c containing murine Fyn. We thank Brendan Cormack for the Mut1GFP construct. We thank Constantine Kreatsoulas for help with GRASP. We thank Mike Eck for the coordinates of c-Src. We thank Suzanne Walker and Dan Kahne for use of their peptide synthesizer. We thank members of the Shokat lab for helpful discussion and comments on the manuscript.

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