Alice Y. Ting[1,†]
Krista Witte[1,‡]
Kavita Shah[2]
Brian Kraybill[3]
Kevan M. Shokat[3]
Peter G. Schultz[1]
[1] *Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037*

[2] *Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121*

[3] *Department of Cellular and Molecular Pharmacology, University of California at San Francisco, San Francisco, CA 94143*

Phage-Display Evolution of Tyrosine Kinases with Altered Nucleotide Specificity*

Abstract: *The problem of identifying downstream targets of kinase phosphorylation remains a challenge despite technological advances in genomics and proteomics. A recent approach involves the generation of kinase mutants that can uniquely use "orthogonal" ATP analogs to phosphorylate substrates in vivo. Using structure-based design, mutants of several protein kinase superfamily members have been found; robust and general methods are needed, however, for altering the nucleotide specificity of the remaining kinases in the genome. Here we demonstrate the application of a new phage display technique for direct functional selection to the identification of a tyrosine kinase mutant with the ability to use N^6-benzyl-ATP. Our method produces, in five rounds of selection, a mutant identical to the best orthogonal Src kinase found to date. In addition, we isolate from a larger library of kinase mutants a promiscuous clone capable of using many different ATP analogs. This approach to engineering orthogonal kinases, combined with others, will facilitate the mapping of phosphorylation targets of any kinase in the genome.* Biopolymers (Pept Sci) 60: 220–228, 2001

Keywords: *phage display; Src; tyrosine kinase; functional selection; orthogonal kinase; phosphorylation; ATP analog; in vitro evolution; nucleotide specificity*

* This paper is dedicated to Professor Merrifield on the occasion of his 80th birthday.

Correspondence to: Peter G. Schultz; email: schultz@scripps.edu

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[†]*Present address:* Howard Hughes Medical Institute and Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093

[‡] *Present address:* Zyomyx Corporation, 3912 Trust Way, Hayward, CA 94545

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INTRODUCTION

Phosphorylation is one of the most important and universal mechanisms of protein regulation in eukaryotic organisms. Kinases and phosphatases, which constitute an estimated 5% of the human genome,[1,2] play dominant roles in the signaling pathways controlling cell growth, division, differentiation, and migration. Mutations in these enzymes have been linked to many forms of cancer. There has been a great push to map kinase and phosphatase functions in the eukaryotic cell. Unraveling their individual roles, their downstream targets, and the factors that influence their regulation is critical to a complete understanding of cell biology and disease mechanisms, and ultimately to the development of new therapeutic agents.

The problem of identifying downstream targets of kinase phosphorylation remains a challenge despite new technological developments in genomics and proteomics. For example, v-Src, one of the most well-studied kinases, causes the tyrosine phosphorylation of over 50 proteins in vivo, yet it is still unclear which of these proteins are direct substrates of v-Src and which are indirect.[3,4] One major obstacle to solving the problem is that the delicate balance of factors controlling a kinase's localization and activity, and hence substrate specificity, inside the cell cannot be replicated in vitro. Thus the problem must be addressed within the cellular context. Because all kinases use ATP as a phosphate source, an analysis of phosphorylated proteins inside the cell does not reveal which kinases are responsible for the placement of which phosphate groups. If a single kinase could be activated at a particular time without activating other kinases, it would be possible to identify its downstream targets; however, known biological stimuli activate multiple kinases at once. Many of these kinases also have overlapping substrate specificities, further complicating the issue.

A recent technique developed by Shokat et al. involves the generation of kinase mutants that use "orthogonal" analogs of ATP rather than ATP as a phosphate source.[5] The method capitalizes on the fact that ATP specificity can be altered without affecting substrate specificity through mutation of a residue conserved across all protein kinase superfamily members. Thus by replacing a kinase with a variant inside a cell, it is possible to exclusively label the direct downstream targets of the kinase of interest while avoiding the labeling of other kinase substrates—a phosphate-radiolabeled "orthogonal" ATP analog is used as a phosphate source only by the engineered kinase, while other kinases in the cell accept ATP. The generality of this technique has been demonstrated with several Src family kinases, c-Abl, and the serine/threonine kinase JNK.[6–10] In these kinases, a highly conserved active site residue (in all cases a large hydrophobic amino acid) in close proximity to the purine ring of the bound ATP was changed to an alanine or glycine, and these mutants were found to accept enlarged ATP analogs such as N^6-benzyl ATP or N^6-phenethyl ATP. The affinity of these mutants for natural ATP was diminished but not abolished. The altered-specificity kinases were transfected into mammalian cells and used to study the downstream targets of phosphorylation.

The full potential of this approach to mapping kinase targets in vivo can only be realized by developing a strategy for rapidly exploring other mutant protein kinases capable of accepting additional "orthogonal" nucleotide analogs. In some cases, rational design has led to binding of orthogonal ATP analogs such as N(benzyl)ribavirin, yet no functional phosphate transfer was observed.[11] In order to create a general strategy for engineering the nucleotide specificity of any kinase with any nucleotide triphosphate, we developed a phage display method for generating orthogonal kinases and optimizing them for unnatural nucleotide selectivity. By sampling a library of kinase mutants on phage, and employing a selection based on direct capture of phosphorylated substrate linked to the phage coat, we identified a Src kinase mutant identical to the best previously found mutant, as well as a mutant with promiscuous and nonselective nucleotide uptake. Our method shows promise as a general strategy for engineering orthogonal kinases, and may be applicable to other proteins such as biotin and methyl transferases.

MATERIALS AND METHODS

Construction of the XD4 Libraries

Overlap extension polymerase chain reaction (PCR) was used to construct both XD4 (*vide infra*) libraries. Deep Vent polymerase (New England Biolabs) was used in the PCR reactions according to the manufacturer's protocol. For the smaller XD4 library, four synthetic oligonucleotides were used to randomize amino acids 337–339: primer 1 (5′-TTTTTTTT*GGCCCAGCCGGCC*ATGGCCATGGGGAGTAGCAAGAGC); primer 2 (5′-AAGGAAAAAA*GCGGCCGC*CTCAGCGACCTCCAACAC); primer 3 (5′-GAGCCCATCTACATC**NNNNNNNNNN**TACATGAGCAAGGG); and primer 4 (5′-CTTGCTCATGTA**NNNNNNNNN**GATGTAGATGGGCTC). Primer 1 contains an *Sfi*I site, and primer 2 contains a *Not*I site (italicized). Primers 3 and 4 contain the nucleotide sequence changes for randomizing amino acids 337–339 (N = A, G, C, or T). For construction of the larger, DNA-

shuffled XD4 library, primers 1 and 2 were used, in addition to the following: primer 5 (323F 5′-GCATGAGAAGCTG**NNK**CAACTGTACGCAGTCGTG); primer 6 (323R 5′-CACGACTGCGTACAGTTG**MNN**CAGCTTCTCATGC); primer 7 (5′-GCTGGTTCAA**NNK**TACGCAGTCGTGTCGG); primer 8 (5′-GACTGCGTA**MNN**TTGAACCAGCTTCTCATGC); primer 9 (5′-GCTGGTTCAACTG**NNK**GCAGTCGTGTCGG); primer 10 (5′-CCGACACGACTGC**MNN**CAGTTGAACCAGC); primer 11 (5′-GAGCCCATCTAC**NNK**GTCATTGAGTACATGAGC); primer 12 (5′-GCTCATGTACTCAATGAC**MNN**GTAGATGGGCTC); primer 13 (5′-CCCATCTACATCGTC**NNK**GAGTACATGAGCAAGGG); primer 14 (5′-CTTGCTCATGTACTC**MNN**GACGATGTAGATGGGCTC); primer 15 (5′-CATCGTCATTGAG**NNK**ATGAGCAAGGGGAGCC) and primer 16 (5′-GCTCCCCTTGCTCAT**MNN**CTCAATGACGATG). Primers 7 and 8 contain the nucleotide sequence changes to randomize amino acid 325 of XD4 (K = G or T; M = A or C). Primers 9 and 10 randomize amino acid 326. Primers 11 and 12 randomize amino acid 326. Primers 13 and 14 randomize amino acid 338. Primers 15 and 16 randomize amino acid 340. The template used for PCR was GST-XD4 in pGEX-KT.[10] *Sfi*I and *Not*I were used to insert the final PCR products into the phagemid vector pFAB5c.His (a gift from J. Engberg, Royal Danish School of Pharmacy),[15] C-terminal to a lacZ promoter and pelB leader, and N-terminal to the pIII gene. The larger XD4 library was DNA-shuffled one time prior to the first round of selection according to the procedure below.

Production of Phage Particles

The phagemid libraries were transformed into electrocompetent *Eschericia coli* XL1-blue (Stratagene) and grown at 37°C in 2× yeast/tryptone broth supplemented with 100 μg/mL ampicillin and 2% glucose. At an optical density $(OD)_{600}$ of 0.5, engineered acid helper phage[13] was added to a final concentration of 1.5×10^8 colony forming units (CFU)/mL and the culture was incubated at 37°C for 40 min. The cells were pelleted and resuspended in 2× YT 100 μ*M* isopropyl β-D-thiogalactoside 100 μg/mL ampicillin 50 μg/mL kanamycin, and shaken for 14 h at 30°C. Cells were pelleted and phage particles in the supernatant were polyethylene glycol-precipitated, and resuspended in TBS (25 m*M* Tris · HCl, pH 7.4/140 m*M* NaCl/2.5 m*M* KCl). Phage titrations were performed with *E. coli* XL1-blue according to standard procedures.[16]

Phage Selections

Approximately 10^{10}–10^{11} phage particles displaying the XD4 library were incubated in 500 μL TBS/100 μ*M* reduced gluathione/100 μ*M* oxidized gluathione with 5 μ*M* of reduced/purified coiled-coil substrate peptide (NH_2-GEI**Y**GEFGGS$(GGS)_4$*AQLKKKLQALKKKNAQLKWKLQALKKKLAQ*GGC-CO_2H, where the tyrosine to be phosphorylated is boldfaced and the basic coil is italicized) at 30°C for 2 h with shaking. The phage particles were precipitated twice to remove excess peptide, resuspended in 195 μL of kinase reaction buffer (50 m*M* Tris, 50 m*M* NaCl, 10 m*M* $MgCl_2$, pH 7.4), and combined with nucleotide triphosphate at a final concentration of 25 μ*M*. The reaction was incubated at 25°C for 24 h. The phage particles were precipitated twice with polyethylene glycol and resuspended in 400 μL of TBS/0.1% bovine serum albumin (BSA)/0.1% Tween. Biotinylated antiphosphotyrosine antibody (1 μg, Signal Transduction Laboratories) was added and the mixture was incubated with gentle rotation at 4°C for 4–12 h. Streptavidin-coated magnetic beads (25 μL, Boehringer Mannheim) were added to pellet the antibody-phage complex. After rotation for 45 min at 4°C, the beads were washed eight times with 1 mL of TBS/0.1% BSA/0.1% Tween, and two times with TBS/0.1% BSA. The phage were eluted with 50 μL of 100 m*M* glycine pH 2.2, 5 min at 25°C, and neutralized with 5 μL of 2 *M* Tris pH 8.0.

DNA Shuffling Between Rounds of Selection

To shuffle the library, the XD4 gene was amplified by PCR (*Taq*, Promega) using the N- and C-terminal primers 5′-TTTCACACAGGAAACAGC and 5′-AGGAGGTTGAGGCAGGTC, respectively. The PCR product was digested with DNase I in a reaction consisting of 43 μL of the DNA, 5 μL of 10× DNase buffer (200 m*M* Tris, 100 m*M* $MgCl_2$, pH 8.0), and 2 μL of DNase I (a 1:1000 dilution of Sigma DNase I). DNA fragments 50–250 bp in size were purified from the reaction mixture using a 3% agarose gel and resuspended in 100 μL of water. Reassembly of the fragments was accomplished by PCR (*Taq*) without primers using the following cycle: 94°C, 2:30 min; 30 cycles of [94°C, 30 s; 50°C, 1 min; 72°C, 45 s + 2 s/cycle]; 72°C, 5 min; 4°C hold. The reassembled gene was amplified in a second PCR reaction using primers 1 and 2 (above). The enzymes *Not*I and *Sfi*I were used to clone the shuffled XD4 library back into pFAB5c.his.

Characterization of XD4 Mutants

XD4 mutants were characterized by transferring the encoding genes from the phagemid vector to the expression vector pGEX-KT as follows. The gene was amplified by PCR using the primers 5′-TTTGGATCCATGGGGAGTAGCAAGAGCAAG and 5′-TTTGAATTCCTACTCAGCGACCTCCAACAC, digested with *Bam*HI and *Eco*RI, and ligated into similarly digested pGEX-KT. Expression and purification of the XD4 kinase as a fusion to GST was completed according to published procedures.[10] The XD4 mutants were assayed by radioactive phosphocellulose blot,[17] phosphorylation of the protein GFP-IYGEF followed by antiphosphotyrosine Western blot,[18] or competitive inhibition of γ-^{32}P-ATP uptake.[6]

RESULTS AND DISCUSSION

c-Src is a ubiquitous tyrosine kinase involved in a wide variety of signaling processes in eukaryotic cells

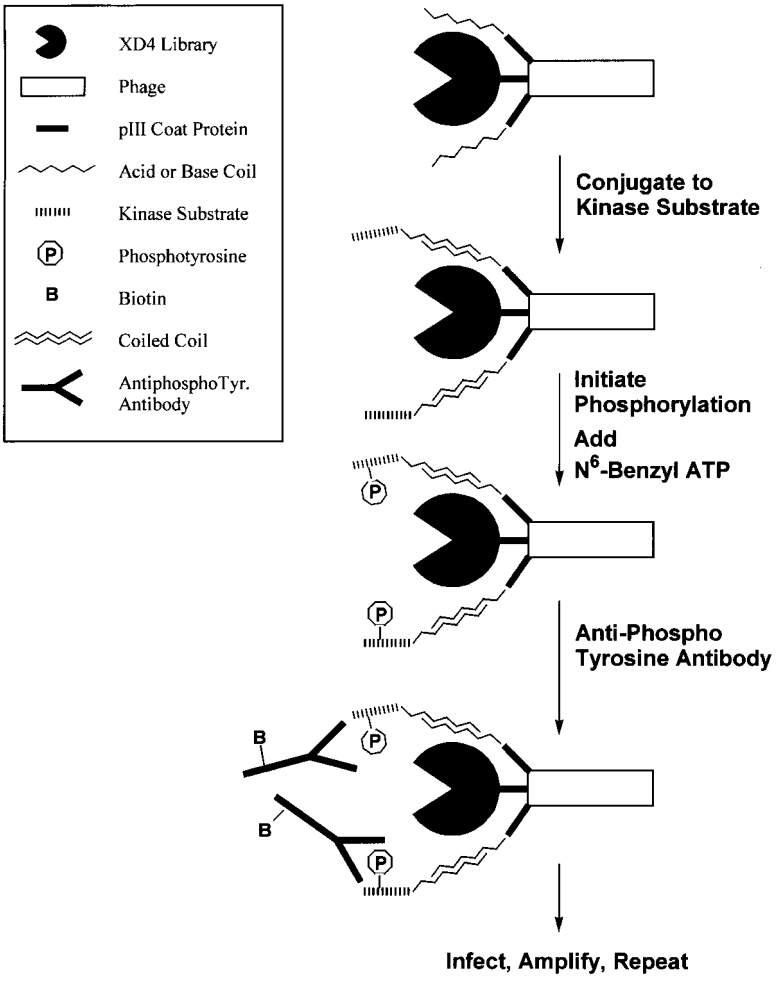

FIGURE 1 General selection scheme used to isolate XD4 mutants with catalytic activity in the presence of N^6-benzyl ATP.

ranging from growth to differentiation. Because it is one of the most well-studied kinases, as well as one of the most frequently mutated proteins in human cancers, there is great interest in identifying the downstream targets of c-Src phosphorylation. To generate a c-Src kinase with altered nucleotide specificity, we prepared a phage library of kinase mutants and selected clones with the ability to use N^6-benzyl ATP instead of ATP.

The starting point for our library was XD4, a constitutively active version of v-Src kinase. The advantages of XD4 for phage selection are its small size (40 kD) and lack of an intact substrate specificity domain (SH2); since we aim to modify nucleotide specificity without changing substrate specificity, it is beneficial to exclude the SH2 domain from the selections. Three active-site residues (337, 338, and 339) were chosen on the basis of their proximity to the purine ring of ATP in the crystal structure[12] and randomized by PCR mutagenesis. The library was cloned into a phagemid expression vector and the phage particles were produced by transforming *E. coli* with the library and superinfecting with an engineered "acid" helper phage.[13]

The selection scheme depicted in Figure 1 was employed to amplify from the library XD4 mutants with the ability to use N^6-benzyl ATP. Due to the special acid helper phage used in the phage production, each phage particle in the library displayed several copies of one half of a coiled-coil fused to the coat protein pIII. To conjugate the tyrosine-containing peptide substrate to the phage, the library was incubated with a peptide consisting of the kinase substrate fused to the other half of the coiled-coil. A disulfide bond between the two termini of the coil sequences resulted in a covalent complex. N^6-benzyl ATP was added to the phage library in reaction buffer to initiate phosphorylation of the conjugated peptide substrate.

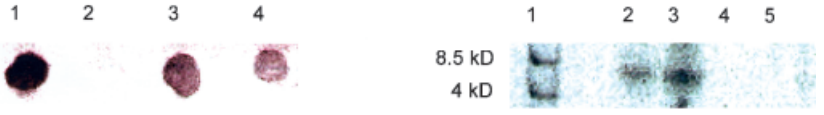

FIGURE 2 The activity of wild-type XD4 on the surface of phage was verified by antiphosphotyrosine Western blot (left) and γ-^{32}P-ATP phosphorylation (right). The dot blot on the left: (1) positive control (2) no ATP control (3) XD4, ATP (4) phage-XD4, ATP. Twenty percent polyacrylamide gel on the right: (1) molecular weight markers (2) phage-XD4, ATP (3) phage-XD4 library, ATP (4) ATP only (5) phage-staphylococcal nuclease (negative control).

After 24 h, the reaction was quenched by precipitation of the phage, and biotinylated antiphosphotyrosine antibody was added to capture phage particles that had been intramolecularly phosphorylated by active kinases. In this selection, only one catalytic turnover is necessary for phosphorylation of the phage molecule and retrieval by antibody capture. Streptavidin-coated magnetic beads were used to pellet the antibody–phage complex, and the particles were rinsed several times with detergent-containing buffer to eliminate nonspecific interactions. The captured phage particles were eluted from the beads with denaturing acidic buffer and amplified in bacteria for further rounds of selection.

To verify the efficacy of the selection scheme and optimize its parameters, two control experiments were performed. First, the activity of the displayed wild-type XD4 enzyme on phage was verified in an activity assay with γ-^{32}P-ATP (100 μM). A steady increase in radiolabeling of conjugated peptide substrate was observed to occur over 24 h at room temperature (Figure 2). Second, the efficacy of the antiphosphotyrosine antibody method of product capture was tested in a control selection with phage displaying peptide substrate that had been chemically phosphorylated. Various amounts of the capture antibody were tested and the best ratio of product over background capture ($\sim$50-fold) was obtained with $\sim$1 μg of antibody per 500 μL of phage solution (data not shown).

The XD4 phage library with amino acids 337–339 randomized was taken through several rounds of selection, and individual clones from each round were isolated and sequenced. There was no detectable increase in phage recovery as the selection progressed. By the fifth round, complete consensus for the sequence (337)–Val–Ala–Glu–(339) was observed (Figure 3). This mutant differs from wild-type XD4 only in the identity of amino acid 338 (Ile338 $\rightarrow$ Ala). Strikingly, it is identical to the best XD4 mutant generated by Shokat et al. for N^6-benzyl ATP uptake using structure-based design.[6,10] The kinetic parameters of this mutant compared to wild-type XD4 are shown in Table I.[4]

It is interesting that from a starting library with a diversity of $64^3 = 2.6 \times 10^5$, only one clone survived to the fifth round of selection. The result suggests either that the Ile338Ala mutant is far superior to any other mutant in the 337–339 library, or that the selection is capable of discriminating between mutants with only small differences in activity. The latter hypothesis is supported by the fact that the k_{cat} for another position 338 mutant, Ile338Gly—a mutant also present in our starting XD4 library—is only 2.5-fold less than that of Ile338Ala for N^6-benzyl ATP uptake (Table I),[6] yet our selection amplifies the Ile338Ala mutant well over the Ile338Gly mutant. Although, as mentioned above, an active clone needs to catalyze only one phosphorylation event in order to be recognized by the capture antibody, there are multiple phosphorylation substrates attached to each phage molecule. The acid helper phage generates three to five copies of coiled-coil-fused pIII protein at one end of each phage particle. Hence an XD4 mutant with higher catalytic activity than another could in principle phosphorylate more of the attached tyrosine peptides on the phage coat and improve its chances of retrieval by the antiphosphotyrosine antibody through multivalent capture. In future experiments, we will vary the length of time of the phosphorylation reaction, as well as the nucleotide triphosphate concentration, in order to examine this hypothesis.

One of the advantages of a combinatorial approach to protein engineering is that it is possible to find amino acids important to selectivity that are distant from the active site and would not be targeted for

Table I Kinetic comparison of wild-type XD4 and mutants

	ATP		N^6-Benzyl-ATP	
	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)
WT XD4	2	12	—	—
I338A XD4	1	70	0.5	20
I338G XD4	0.8	80	0.2	5

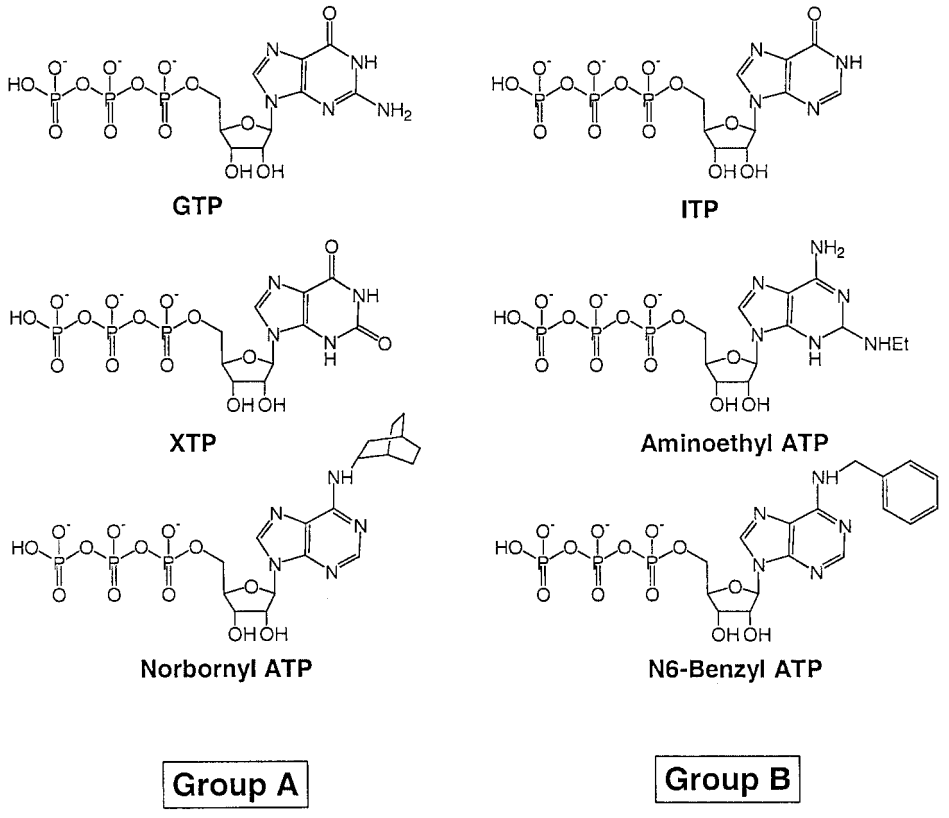

FIGURE 4 Groups of ATP derivatives used in the selections.

mutagenesis by rational design. We set out to optimize the selectivity of the XD4 Ile338Ala mutant by preparing such a library of kinase mutants with mutations dispersed across the entire gene, and employing the technique of DNA shuffling to combine advantageous mutations with one another and further diversify the library between rounds of selection.[14] We also screened the library against a panel of five different ATP analogs in addition to N^6-benzyl-ATP (Figure 4). The larger kinase library was prepared as follows. Individual active site residues in XD4 (323, 325, 326, 336, 338, 340), selected on the basis of their proximity to the purine ring of ATP (Figure 5),[12] were randomized one by one using overlap extension PCR. The resulting small libraries were combined to form a single large library in which each member had a single mutation at one of six active site amino acids. The combined library was then DNA-shuffled one time prior to the first round of selection in order to cross mutations and generate randomizations across the entire gene. Characterization of the library by sequencing indicated an average mutation rate of two amino acids per library member.

The phage particles were generated in the same manner as before, using the engineered acid helper phage, and subjected to the same selection scheme as

Wild type:

```
TAC ATC GTC ATT GAG TAC ATG
        Val Ile Glu
```

Library:

```
TAC ATC NNN NNN NNN TAC ATG
```

Round 5:

```
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
TAC ATC GTC GCT GAG TAC ATG
        Val Ala Glu
```

FIGURE 3 Consensus sequence after five rounds of selection with the XD4 library. This mutant differs from the wild-type only in the identity of amino acid 338 (Ile338 → Ala) and is identical to the best XD4 mutant found by Shokat et al. using rational engineering for N^6-benzyl ATP uptake.[6,10]

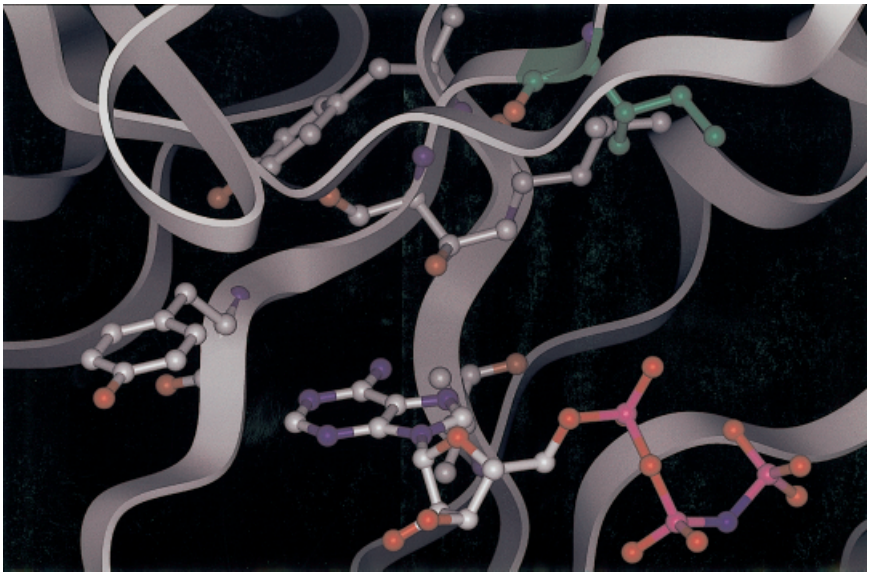

FIGURE 5 Active site of c-Src complexed with the non-hydrolyzable AMP-PNP.[15] The six residues randomized for the larger XD4 library are rendered in ball and stick format. Residue 336, the amino acid that when mutated to arginine eliminates ATP specificity, is colored green.

shown in Figure 1. Two groupings of nucleotide analogs were used in the selections (Figure 4). Group A nucleotides consisted of GTP, XTP, and N^6-norbornyl ATP. Group B consisted of ITP, N^6-benzyl ATP, and 2-aminoethyl ATP. After three consecutive rounds of selection with DNA shuffling in between each round, several clones were taken from each selection group and sequenced. The results are listed in Figure 6. There was no detectable increase in phage recovery over the three rounds.

After three rounds of selection, the mutations were centered at residue 336, although no amino acid consensus was observed. However, the mutant Ile336Arg appears five times among the sequences and was selected for expression and characterization. The gene encoding this mutant was transferred from the phagemid vector to an expression vector, and the enzyme was expressed and purified as a fusion to gluathione S-transferase (GST). XD4 Ile336Arg was highly active in a peptide phosphorylation assay with γ-^{32}P-ATP, and dissimilar to wild-type XD4 in its nucleotide specificity according to a GFP-Western blot assay[18] and a γ-^{32}P-ATP competition assay. As shown in Figure 7, XD4 Ile336Arg is a promiscuous kinase, capable of using all the ATP derivatives tested to a fairly equal extent.

According to the crystal structure of c-Src complexed to ADP,[12] the side chain of residue 336 points away from the active site and contacts amino acids of the second shell surrounding the active site. By con-

% Inhibition

Nucleotide	WT XD4	I336R XD4
ATP	80 %	83 %
N6-Benzyl ATP	5 %	97 %
GTP	10 %	79 %
ITP	4 %	61 %
XTP	7 %	71 %
N6-Norboryl ATP	10 %	75 %

FIGURE 7 Nucleotide specificity assay for WT XD4 and the Ile336Arg mutant. The percent inhibition of ATP usage was measured in the presence of the nucleotide derivatives listed in the table. Use of γ-^{32}P-ATP (10 μM) by WT XD4 was inhibited by only ATP, whereas use of γ-^{32}P-ATP by XD4 I336R was inhibited to a high degree by all the nucleotide derivatives tested (at 100 μM), indicating that XD4 I336R is a promiscuous, nonselective kinase.

verting Ile to Arg, it is possible that this mutant introduces new steric bulk to the interface between the first and second shells, thereby causing indirect distortion of the active site. The normally very specific active site, now significantly reorganized, accommodates many different nucleotide shapes.

There are several reasons why a nonselective kinase could have been favored by our selection conditions. First, no negative selection was implemented to remove mutants with the ability to still use ATP. Second, differences in expression level can bias representation in the library significantly. We observed that the soluble form of Ile336Arg XD4 was highly expressed in bacteria; whether or not this also implies high expression by phage is unclear. Third, by grouping together different nucleotide analogs for the selections, we perhaps biased the library toward mutants with structurally permissive active sites. Future experiments will address all of these points, as well as sample other kinds of kinase libraries, such as a library derived from Ile338Ala XD4 (the best orthogonal mutant to date) and libraries derived from mutants unable to use ATP.[11]

CONCLUSIONS

Challenges in protein engineering have increasingly been met by combinatorial methods, which confer numerous advantages over rational design. Phage display is one such powerful technique, whose versatility is enhanced when it is coupled with novel methods for direct selection of enzyme function. Here we demonstrate the application of a new phage technique for direct functional selection to the identification of a tyrosine kinase mutant with the ability to use N^6-benzyl-ATP. This approach to engineering orthogonal kinases, combined with other approaches, will facilitate the mapping of phosphorylation targets of any kinase in the genome. It may also be applicable to altering the specificity of other enzymes including methyl and biotin transferases.

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323     340    336
|       |      |
VQLYAVVSEEPIYIVIEYMSK
```

Third Round, Group A	Third Round, Group B
VQL**V**AVVSEEPIYIVIEYMSK	VQLYAVVSEEPIYIVIEYMSK
VQLYAVVSEEPIY**R**VIEYMSK	VQLYAVVSEEPIYIV**P**EYMSK
VQLYAVVSEEPIY**S**VIEYMSK	VQLYAVVSEEPIY**R**VIEYMSK
VQL**S**AVVSEEPIY**R**VIEYMSK	VQLYAVVSEEPIY**A**VIEYMSK
VQL**G**AVVSEEPIYIV**R**EYMSK	VQL**G**AVVSEEPIYIVIEYMSK
VQLYAVVSEEPIYIVIEYMSK	VQ**R**YAV**M**SEEPIY**P**VIEYMSK
VQLYAVVSEEPIY**P**VIEYMSK	VQL**P**AVVSEEPIY**Q**VIEYMSK
VQLYAVVSEEPIY**R**VIEYMSK	VQLYAVVSEEPIYIVIEYMSK
VQL**N**AVVSEEPIYIVIEYMSK	VQLYAVVSEEPIY**V**VIEYMSK
VQLYAVVSEEPIYIVIE**H**MSK	VQLYAVVSEEPIY**T**VIEYMSK
VQLYAVVSEEPIY**W**VIEYMSK	VQLYAVVSEEPIY**G**VIEYMSK
VQLYAVVSEEPIY**W**VIEYMSK	VQLYAVVSEEPIY**R**VIEYMSK
VQLYAVVSEEPIYIVIE**C**MSK	VQLYAVVSEEPIY**P**VIEYMSK
VQL**R**AVVSEEPIYIVIEYMSK	VQLYAVVSEEPIY**L**VIEYMSK
VQLYAVVSEEPIY**L**VIEYMSK	VQLYAVVSEEPIY**Y**VIEYMSK
VQLYAV**A**SEEPIYIVIEYMSK	VQLYAVVSEEPIYIVIE**F**MSK
VQLYAVVSEEPIYIVIEYMSK	

FIGURE 6 Results of sequencing after three rounds of selection with two different nucleotide groups. The library was shuffled in between rounds. The amino acids that differ from the wild-type sequence (shown at top) are boldfaced and underlined. After the third round of selection, the mutations appeared to be centered at position 336, although there was no clear consensus in amino acid identity at this site.

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