Src–Abl Tyrosine Kinase Chimeras: Replacement of the Adenine Binding Pocket of c-Abl with v-Src To Swap Nucleotide and Inhibitor Specificities†

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ABSTRACT: Engineered protein kinases with unnatural nucleotide specificity and inhibitor sensitivity have been developed to trace kinase substrate targets. We first engineered unnatural nucleotide specificity into v-Src by mutating one residue, isoleucine 338, to alanine. This position is highly conserved among all kinases in the sense that it is always occupied by either a large hydrophobic residue or threonine. Because of the conservation of this residue and the highly conserved fold of the kinase family, we have attempted to generalize the engineering of all kinases on the basis of our success with v-Src. Although many kinases can be similarly engineered using v-Src as a blueprint, we encountered one kinase, c-Abl, which when mutated, does not display the ability to accept unnatural ATP analogues. To overcome this failure of the engineered c-Abl (T315A) to accept unnatural nucleotides, we developed a new strategy for introducing unnatural nucleotide specificity into kinases. We generated a chimeric kinase in which regions of the kinase domain of c-Abl were swapped with the corresponding regions of v-Src (I338A). Specifically, we engineered two chimeras in which the N-terminal lobe of the SH1 domain of c-Abl was swapped with that of v-Src. These kinase chimeras were found to have the same unnatural nucleotide specificity as that of v-Src (I338A), while retaining the peptide specificity of c-Abl. Thus, these chimeric kinases are suitable for identifying the direct substrates of c-Abl. These engineered chimeric enzymes provide a new strategy for constructing kinases with tailor-made ligand binding properties.

The protein kinase superfamily is one of the largest (1000–2000 members) eukaryotic protein families identified to date (1–3). The phosphorylation of tyrosine, serine, and threonine residues plays a central role in almost every known signal transduction cascade in eukaryotic cells. The identification of the cellular substrates of individual protein kinases remains one of the central challenges in the field (4, 5). We have developed a chemical genetic method for uniquely tagging the direct substrates of protein kinases (4, 5). This method relies on protein engineering to create a kinase that catalyzes an unnatural phospho-transfer reaction. The engineered kinase utilizes an ATP analogue that is not a substrate for any wild-type protein kinase (an orthogonal ATP analogue). By utilizing a γ-32P-labeled form of the ATP analogue, the direct substrates of the mutant kinase become uniquely radiolabeled even in the presence of all other wild-type protein kinases.

We first identified a single amino acid in the protein kinase v-Src,1 residue I338, which controls the recognition of N6-substituted ATP analogues (4, 5). When this residue is mutated to alanine or glycine, the mutant v-Src kinase can accept ATP analogues with large substituents at the N6 position of ATP (termed A*TPs). Sequence analysis of the kinase superfamily revealed that the residue corresponding to I338 in v-Src is always occupied by a large hydrophobic residue (leucine, isoleucine, glutamine, methionine, or phenylalanine) or by threonine (5). This analysis suggested that similar alanine or glycine mutations (at the residue corresponding to I338 in v-Src) would have the desired effect on the nucleotide (ATP/A*TP) specificity of any protein kinase in the genome. Indeed, mutation of the residue corresponding to position 338 (in v-Src) to alanine or glycine confers unnatural nucleotide and inhibitor specificity to other Src family tyrosine kinase members as well as to many serine/threonine protein kinases (4–8).

The fact that the v-Src (I338) residue is so well conserved (Figure 1) among protein kinases suggested there might be some structural importance to this residue. Conserved residues within protein families have been found to be important predictive elements of protein structure and function (9–11). As we extended our chemical genetic method to the study of different kinases, we expected that...
some protein kinases would not tolerate this space-creating mutation; i.e., some kinase mutants might lose significant catalytic activity or not be capable of catalyzing A*TP-dependent phospho-transfer reactions. c-Abl is one such kinase in which the single mutation did not produce an enzyme capable of catalyzing A*TP-dependent phosphorylation.

c-Abl has been identified as the cellular homologue of the v-Ab1 oncogene of the Abelson murine leukemia virus, which causes leukemia in mice (12). c-Abl is a key cellular tyrosine kinase that is involved in cell cycle regulation, acts as a growth activator, controls signaling events in the cytoplasm, and has been found to be critical for entry of cells into the S-phase of the cell cycle (13, 14). c-Abl is also the only tyrosine kinase known to have a DNA binding domain and is activated in response to extracellular stimuli such as ionizing radiation and other DNA-damaging agents (15). Additionally, c-Abl has also been identified as the target of a genetic translocation in the Philadelphia-chromosome positive human leukemia in which the c-Abl gene is fused by a translocation event between chromosomes 9 and 22 with the mammalian break point cluster region (BCR) gene (16). Identification of the direct cellular substrates of c-Abl would allow for delineation of the signaling pathways activated by this kinase.

c-Abl and v-Src both have conserved SH3, SH2, and SH1 (catalytic) domains (Figure 2). c-Abl differs from v-Src in that the C-terminus of c-Abl contains a DNA binding domain, an actin-binding domain, a nucleotide localization signal, and proline-rich sequence which influences subcellular localization, interactions with specific proteins and DNA targets, and substrate recruitment (14). Although no structural information is currently available for the c-Abl tyrosine kinase catalytic domain, all existing crystal structures of kinase catalytic domains have exhibited a conserved two-lobe structure. The smaller SH1 domain N-terminal lobe is primarily responsible for binding the protein substrate (17). The interface between the two lobes forms a cleft in which ATP binds (18–21). Crystal structures of Src family kinases show that the residue corresponding to v-Src (I338) falls in a hinge region of the kinase domain that links the N-terminal and C-terminal lobes (4, 5, 19, 20, 22). This position is indicated by the asterisk (*) in Figure 2.

To engineer unnatural nucleotide specificity into c-Abl, we attempted to use our successful engineering of v-Src as a blueprint. The sequence of the catalytic domain (SH1) of c-Abl is 50% identical and 68% homologous to the sequence of the SH1 domain of v-Src. Despite the close relatedness of these two proteins, the alanine mutation in c-Abl (residue T315 in c-Abl) corresponding to I338A in v-Src did not allow c-Abl to accept ATP analogues. To create a suitable mutant of c-Abl that would accept orthogonal ATP analogues, we developed a new strategy for altering kinase nucleotide specificity. We took advantage of v-Src’s ATP binding site specificity by engineering it into c-Abl. Since the kinase domain N-terminal lobe is primarily responsible for ATP recognition (and in our case A*TP recognition), we decided to graft the SH1 domain N-terminal lobe of v-Src (I338A), which accepts A*TPs, onto the SH1 domain C-terminal lobe of c-Abl, thereby forming a chimeric protein tyrosine kinase (Figure 3). We asked whether a chimeric kinase constructed by grafting c-Abl and v-Src together within the SH1 domain could be utilized as a means of engineering unnatural nucleotide specificity into c-Abl. The retention of c-Abl’s peptide substrate specificity in the chimeric kinase is critical to the successful use of the kinase substrate tagging strategy.

**MATERIALS AND METHODS**

**Construction of Chimeras.** Chimeras ASA, SA, AS, and AS2 were constructed using overlap extension PCR (23). Pfu polymerase (from Stratagene) was used according to the manufacturer’s protocol. Eight oligonucleotides used to generate the chimeras were as follows: P1, 5′-TTTGGATCC-CAACAGCCTGGGACAAAATTCC; P2, 5′-TTTGAATTCC-

**FIGURE 1:** Sequence alignment of residues surrounding v-Src (I338) in protein tyrosine and serine/threonine kinase catalytic domains. Residue v-Src (I338) and the corresponding residue in other kinases are indicated by the arrow. The numbering of the starting residue is listed in parentheses before each sequence. The kinases are as follows: c-Src, cellular homologue of Rous sarcoma virus tyrosine kinase; v-Src, viral homologue of c-Src; c-Abl, cellular homologue of Abelson murine leukemia virus; Lck (p56Lck), lymphoid T-cell protein tyrosine kinase; Fyn (p59Fyn), proto-oncogenic tyrosine kinase; Fn, p, protein kinase related to Fgr and Yes; Hck, hematopoietic cell protein tyrosine kinase; PKA (cAMP), cyclic AMP-dependent protein kinase; Cdk2, type two cyclin-dependent protein kinase; CaMKII, calcium- and calmodulin-dependent protein kinase; APH or APH(3′)-IIIa, aminoglycoside kinase.

**FIGURE 2:** (A) Domain structures of c-Abl, v-Src, and SH1 domain constructs (the fragment of v-Src known asXD4 and the truncated version of c-Abl) used in this study. The domain abbreviations are as follows: BD, binding domain; NLS, nuclear localization signal; SH1, Src homology 1 domain (the catalytic domain); SH2, Src homology 2 domain; SH3, Src homology 3 domain. The site of our mutation, v-Src (I338) and c-Abl (T315), is indicated by the asterisk. (B) Constructed chimeric protein kinases used in this study. The chimeras are designated ASA, SA, AS, and AS2 for Ab1—Src—Ab1, Src—Ab1, and Ab1—Src (two versions), respectively.

**FIGURE 3:** Amino acid sequence information of the catalytic domain (SH1) of c-Abl, v-Src (two versions), respectively. BD, binding domain; NLS, nuclear localization signal; SH1, Src homology 1 domain (the catalytic domain); SH2, Src homology 2 domain; SH3, Src homology 3 domain. The site of our mutation, v-Src (I338) and c-Abl (T315), is indicated by the asterisk. The numbering of the starting residue is listed in parentheses before each sequence. The kinases are as follows: c-Src, cellular homologue of Rous sarcoma virus tyrosine kinase; v-Src, viral homologue of c-Src; c-Abl, cellular homologue of Abelson murine leukemia virus; Lck (p56Lck), lymphoid T-cell protein tyrosine kinase; Fyn (p59Fyn), proto-oncogenic tyrosine kinase; Fn, p, protein kinase related to Fgr and Yes; Hck, hematopoietic cell protein tyrosine kinase; PKA (cAMP), cyclic AMP-dependent protein kinase; Cdk2, type two cyclin-dependent protein kinase; CaMKII, calcium- and calmodulin-dependent protein kinase; APH or APH(3′)-IIIa, aminoglycoside kinase.
ligated into the Bam of the ASA gene was digested with PCR. Then fragments 4 and 2 were gel purified and fused together to form fragment 4 using overlap extension. Fragments 1 and 3 were gel purified and fused together to form the chimera gene ASA. The PCR product was then fused to form the chimera SA gene. The AS chimera was created using primers P1, P3, P4, and P8. The AS2 chimera was created using primers P1, P5, P6, and P8. The following construct was used for our single-mutant kinase studies: GST−ΔSH3 c-Abl, which contains the SH2 and SH1 domains of c-Abl but has the SH3 domain deleted, was fused to GST. The c-Abl (wild-type) and c-Abl (T315A/G) mutants in this paper refer to this truncated c-Abl construct. All kinases were sequenced over the entire coding region (oligonucleotide sequencing and synthesis facility, Princeton University).

Protein Expression and Purification. The kinases were expressed in E. coli DH5α cells using the pGEX-KT vector system (Pharmacia). Purification of the GST-fused kinase was carried out as described by Xu et al. (24) with slight modifications. Briefly, 25 mL of LB-superbroth medium was inoculated with a single colony, grown overnight at 37 °C in a shaker, and then transferred into 250–750 mL of LB-superbroth (Bio 101) and grown to a cellular density A600 of 0.5–0.7 (approximate time of 2 h). Protein expression was then induced with IPTG (0.1 mM) for 3–5 h, and the culture was then stored overnight at 4 °C before cell lysis. Storage at 4 °C overnight is essential for producing highly active kinases (see below) (4, 8). Cells were lysed with a French press [the cell pellet was resuspended in 25 mM Tris (pH 8.0), 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT before lysis]. The GST fusion proteins were then purified on immobilized glutathione–agarose beads (Sigma), and the protein kinase bead slurry was washed three times with 25 mM Tris (pH 8.0), 10 mM EDTA, and 100 mM NaCl, followed by two washes with 50 mM Tris (pH 8.0). The GST fusion proteins were then eluted from the resin with free glutathione [freshly prepared 5 mM reduced glutathione in 50 mM Tris (pH 8.0)] and used as such. More active kinases (Table 2) were obtained after the affinity-purified kinases were concentrated in Centricon-10 concentrators (Amicon), with a final isolated yield of 250 μg/L of culture. The protein purity and quantity were determined via silver stain SDS–PAGE methods using the Gelcode silver stain kit (Pierce).

Peptide Substrate Synthesis and Characterization. Short peptide substrates for v-Src and c-Abl were synthesized via solid-phase peptide synthesis on an ABI431A peptide synthesizer (25). Fmoc-protected amino acids and WANG resins (NovaBiochem) were used (26, 27). The Fmoc procedure (HBTU/HOBT) was used for coupling (26). Peptides were cleaved with a TFA/thioanisole/EDT/H2O mixture. The peptide sequence was confirmed via Edman degradation sequencing and the molecular ion by mass spectrometry. The purity (>97% pure) was determined by reverse-phase HPLC on a C18 column. The following mass spectra data (Hewlett-Packard 5989B electrospray mass spectrometer) were obtained for the peptides that were studied: EYGEFKKK (Src family kinase optimal substrate), expected mass of 1141.33 and molecular ion of 1140.85; IYGEFKKK, expected mass of 1012.22 and molecular ion of 1012.8; EAIYGEFKKK, expected mass of 1212.41 and molecular ion of 1212.9; EYWEFKKK, expected mass of
1270.49 and molecular ion of 1270.7; YIYGSFKKK, expected mass of 1336.36 and molecular ion of 1334.8; GIYWHHYKKK, expected mass of 1359.60 and molecular ion of 1359.26; ESLYWSWPKKK, expected mass of 1451.69 and molecular ion of 1452.2; NIYGHFKKK, expected mass of 1314.35 and molecular ion of 1315.0; KVEKIGETGYGVYVK (from p34cdc2, sequence IYGEF), expected mass of 1669.94 and molecular ion of 1670.6; EAIYAAPKKK, expected mass of 1647.26 and molecular ion of 1046.90; and EAIYAAPFKKK (Abl optimal substrate), expected mass of 1336.60 and molecular ion of 1337.

In Vitro Peptide Assay for Kinase Activity. Phosphorylation assays of the kinases with peptide substrates were carried out in triplicate at 22 °C in a final volume of 30 μL containing 50 mM Tris, 10 mM MgCl₂, 1 mg/mL BSA, 0.1 mM peptide with 1-2 nM kinase, and 10 μM [γ-32P]ATP (1000 cpm/pmol) (DuPont/NEB), buffered at pH 8.0. For inhibition studies, unlabeled ATP or A*TP analogues (analogues 1-3) (100 μM) were added prior to the addition of the enzyme. After 30 min, the reactions were quenched by spotting 25 μL of the reaction volume onto p81 phosphocellulose disks (Whatman) and immersing in 50 mL of 10% acetic acid for >30 min, followed by washing three times in 50 mL of 0.5% phosphoric acid and one time in 50 mL of acetone. Scintillation counting according to standard methods was performed.

Kinase Kinetics Assay. The kinase reactions were performed in triplicate in kinase reaction buffer [50 mM Tris (pH 8.0), 10 mM MgCl₂, and 100 mM NaCl]. [γ-32P]ATP (~1000 cpm/pmol) was added to the wild-type kinases, while the [γ-32P]A*TPs (1000 cpm/pmol) were used for studies with the mutant kinases. For the determination of peptide kinetics, the final concentration of ATP or A*TP was kept at 100 μM. The reaction progress is linear for 30 min and linear with respect to the enzyme concentration. After 30 min, the reaction mixture was spotted onto phosphocellulose (p81) paper and washed for 30 min with 10% acetic acid, followed by three 10 minute washes with 0.5% phosphoric acid, and a final 1 min wash with acetone. The peptides studied here contain a polylysine tail at the C-terminus to allow for binding of the peptides to the negatively charged phosphocellulose paper disks during the washing steps. Liquid scintillation counting was used to measure the extent of peptide phosphorylation (counts per minute).

**RESULTS AND DISCUSSION**

Chimeric Enzymes. In this study, we extend the utility of chimeric proteins by constructing chimeric kinases that are capable of accepting unnatural A*TP analogues. Previous studies of chimeric enzymes include the work of Hopfner et al. who combined subdomains of two proteases with different substrate specificity profiles from the S1 serine protease family (30). Nixon et al. combined subdomains of two enzymes catalyzing different chemical transformations (31). Brunet et al. (32) created chimeric proteins composed of two mitogen-activated protein kinases (MAPKs), p38 and p44, which transduce stress and growth factor signals, respectively. In another chimeric protein example involving the MAP kinases, English et al. (33) studied the substrates and regulators of the MAP kinase ERK5. The signals required to activate ERK5 as well as the substrates of ERK5 are unknown, whereas ERK2 is a well-studied MAP kinase, with known activators. Chimeras were constructed between ERK5 and ERK2, consisting of the N-terminal lobe of ERK2 and...
the C-terminal lobe of the ERK5 kinase domain. ERK2 activators can activate this chimera, and the substrates of the ERK2–ERK5 chimera are thought to be the true substrates of ERK5. In a chimera example involving tyrosine kinases, Mathey-Provot et al. characterized v-Abl and v-Src chimeras made by combining v-Abl and v-Src at a point seven residues downstream of the catalytic lysine within the kinase domain (34). The catalytic lysine in v-Src is residue K295 and in c-Abl is K271 (see Figure 4). The v-Src–v-Abl recombinant protein (v-SBL) has the transformation properties of v-Abl in a variety of cell types. From these results, the phospho-acceptor specificity of the kinase appears to be controlled by the protein sequence following the catalytic lysine. This study suggests that part, but not all, of the N-terminal domain of v-Abl can be swapped with v-Src without affecting the function (as assayed by transformation phenotype) of v-Abl. These examples demonstrate the utility of using chimeras composed of different parent enzymes to create proteins with new properties, and the utility of these chimeras in the manipulation of complex biological systems.

**Protein Engineering of c-Abl Using the Engineered I338G v-Src as a Blueprint.** The protein residue of v-Src (I338) corresponds to T315 in c-Abl and is the engineered position mutated to alanine and glycine (see Figure 1). Wild-type c-Abl, wild-type v-Src (XD4), the single mutants, and the chimeric kinases were expressed and purified as described in Materials and Methods. We chose to express the mutant kinases in bacteria because of the ability to more rapidly make mutations and assay for activity and specificity, rather than using Sf9 insect cell expression systems that require multiple cloning and virus production steps for each desired mutant. One requirement for obtaining highly active kinases from *E. coli* was storage of the bacterial cell culture at 4 °C overnight prior to cell lysis (4, 8). SDS–PAGE analysis of total cell lysates stored at 4 °C overnight compared to those immediately following the 5 h IPTG induction period shows no observable changes in any of the Coomassie-stained protein bands (data not shown). However, silver stain SDS–PAGE analysis of the purified proteins did show an increase (approximately 4-fold) in the amount of purified kinase after 4 °C storage (data not shown). Overnight storage of the cell pellet could be required for expression of active forms of the kinases here due to (1) a chaperone-assisted increase in the level of protein expression via heat shock proteins (HSPs) thought to assist protein folding as reported by Cole or (2) the continual slow induction of protein production as the cell culture cools (35). HSPs have been shown biochemically and genetically to interact directly with kinases in signaling cascades; thus, their involvement in achieving high levels of protein expression from bacteria is not unexpected since the HSPs are conserved between prokaryotes and eukaryotes (36–40). A protein gel of the purified tyrosine kinases investigated in this study is shown in Figure 5.

![Figure 4: Sequence alignment of the v-Src and c-Abl kinase domains. The residues that differ between c-Src and c-Abl are highlighted in gray. The insertions are indicated with arrowheads, and the site of our mutation is shown in v-Src (I338) and c-Abl (T315). The bar represents residues G275–F349 (in v-Src) corresponding to the region of v-Src that is swapped into c-Abl to construct the ASA chimera.](link)

![Figure 5: SDS–PAGE (12%) of the purified kinases used in this study: lane 1, XD-4 construct of wild-type GST–v-Src; lane 2, SA (GST–Src–Abl) chimera; lane 3, ASA (GST–Abl–Src–Abl) chimera; and lane 4, wild-type GST–c-Abl. The protein bands are silver stained according to standard techniques.](link)
not shown). This indicates that the unnatural A*TP substrates (1–3) do not bind effectively to the engineered active site and suggests that they will not be efficient phospho donors (5). The catalytic efficiency of c-Abl (T315A) with N6-(benzyl)-ATP was extremely low. The $k_{cat}/K_M$ of c-Abl (T315A) with N6-(benzyl)-ATP is $<4 \times 10^2$ min$^{-1}$ M$^{-1}$ compared to that of wild-type c-Abl with ATP (2.09 $\times 10^6$ min$^{-1}$ M$^{-1}$) (Table 1). This loss of almost 4 orders of magnitude in catalytic efficiency upon engineering c-Abl to accept unnatural A*TPs does not satisfy one of the critical design criteria we established for engineering kinases to accept unnatural ATP analogues. The primary sequence differences between the ATP binding sites of c-Abl and v-Src are subtle (Figure 4) yet are significant enough to cause the vastly different kinetic behavior of the c-Abl (T315A) and v-Src (I338A) mutants toward A*TP analogues.

**Protein Engineering Design of v-Src and c-Abl Involving Deletion Mutants.** We next looked for second site revertants or other determinants of analogue specificity in addition to the c-Abl (T315A) point mutation for engineering unnatural nucleotide specificity into c-Abl. Comparison of the catalytic domain sequences of c-Abl and v-Src led to the identification of two insertions in the c-Abl catalytic domain N-terminal lobe. One insertion is three residues before the catalytic lysine, while the other insertion is four residues before the T315 position (see Figure 4). We asked if these insertions were responsible for the different kinetic behavior of the engineered c-Abl and engineered v-Src kinases. When these insertions were deleted, the c-Abl deletion mutants, c-Abl-ΔY283, T315A) and c-Abl(ΔP329, T315A), showed no activity even with [γ-32P]ATP (data not shown). We conclude that the residues surrounding the deletion sites must be critical for the structure of the active site; therefore, we cannot make even single residue deletions without making further changes to the c-Abl kinase domain. Rather than continue in an effort to identify suitable second site mutations in the c-Abl (T315A) mutant in an attempt to locate enzymes displaying efficient catalysis with N6-(benzyl)-ATP as a phospho donor, we asked whether we could excise a large stretch of the v-Src (I338A) kinase domain sequence and insert it into c-Abl to recapitulate the desired nucleotide active site specificity (32, 34).

**Protein Engineering Design of v-Src and c-Abl Involving Chimeric Kinases.** A critical design goal for these chimeric kinases is that they use an A*TP with high catalytic efficiency while retaining the protein substrate specificity of c-Abl. The crystal structures of protein kinases with efficiency while retaining the protein substrate specificity of c-Abl contain sequence elements from both v-Src and c-Abl, must contain the N-terminal region of SH2 and SH1 domains from full-length c-Abl (Figure 2A) (42). The majority of the residues that are different between v-Src and c-Abl SH1 domains (50% overall level of sequence identity) are in the C-terminal peptide substrate-binding region. The two wild-type kinases have been shown to have distinct peptide substrate preferences (43). Since the crystal structure of c-Abl has not been determined, we created a c-Abl catalytic domain structure using the homology modeling program Modeller4. Recently, one such homology model of the catalytic domain of v-Abl based on the crystal structure of the insulin receptor kinase (IRK) has been published (44). The high level of sequence identity between the v-Src and c-Abl catalytic domains (Figure 4) paired with the modeling studies suggested that active site mutations in v-Src should be able to be transferred to c-Abl with highly predictable outcomes.

**Nucleotide Recognition via the Kinase Catalytic Domain.** ATP is bound in the cleft formed at the interface of the N- and C-terminal lobes of the kinase SH1 domain. Consequently, residues from both lobes form interactions with the nucleotide substrate (18–20). It is conceivable that alteration of the N-terminal lobe of the SH1 domain alone would not be sufficient to achieve the A*TP recognition properties desired in the chimeric protein kinases. It is known that residues in the N-terminal lobe of the SH1 domain carry out several key catalytic functions, and include the glycinerich loop, which is involved in triphosphate binding and catalysis, as well as the highly conserved catalytic lysine, which is crucial for kinase activity (45–47). The presence of residues critical for catalysis as well as ATP recognition in the N-terminal lobe of the SH1 domain makes engineering nucleotide recognition without loss of catalytic activity a challenge.

**Chimeras Joining v-Src and c-Abl Tyrosine Kinases.** Since the boundary between the two parent enzymes is within the SH1, or catalytic domain, of the protein, the construction of active chimeric kinases requires the careful selection of the junction point. Hopfner et al. (30) constructed an active chimera between coagulation factor Xa and trypsin by choosing a linker region between the N- and C-terminal domains of the protein as the chimera boundary. We utilized this precedent and selected the hinge region linking the N- and C-terminal lobes of the SH1 domain as the boundary between the v-Src and c-Abl sequences. By engineering the boundary position into a loop, we hoped to introduce minimal disruption into the interface of the two lobes and therefore into the kinase active site.

**Catalytic Activity of Chimeras Composed of v-Src and c-Abl Tyrosine Kinases.** The SA (Src→Abl) chimera contains the N-terminal region of v-Src and the C-terminal region of SH2→SH1 c-Abl (Figure 2B). The AS and AS2 chimeras contain the N-terminal region of SH2→SH1 c-Abl grafted onto the C-terminal region of v-Src. The fusion site in the AS2 chimera is in the beginning of the glycine-rich loop (G275 corresponding to v-Src), and the fusion site in the AS chimera is at the end of the hinge (corresponding to F349 in v-Src); the equivalent junction point (F349) is also used in the SA chimera. The ASA “triple” chimera was constructed by swapping the region of residues G269–Y345 in SH2→SH1 c-Abl with the corresponding region of residues G275–F349 from v-Src (Figure 2B).

Kinetic characterization of the chimeras was carried out using peptide phosphorylation assays (Table 1) (48). The SA chimera exhibited activity similar to that of c-Abl (T315A) with ATP as the phospho donor. The ASA chimera displays 32% of the catalytic efficiency of c-Abl (T315A). Significant differences in protein stability (measured by loss of kinase activity when stored at −80 °C) were observed between the
chimeric kinases. Both SA and ASA chimeras were as stable as v-Src (I338A) and more stable than c-Abl (T315A). The AS and AS2 chimeras were not only much less active than v-Src or c-Abl but also extremely unstable and lost their kinase activity within several hours of purification. The AS and AS2 chimeras displayed 20 and 40% of the activity of the wild-type kinases, respectively, with ATP as the phospho donor. The percent activity of the chimeric kinases relative to the wild-type kinases was determined by simultaneous purification of the wild-type and chimeric kinases and was calculated as a ratio of $V_0$ (counts per minute in the presence of mutant kinase)/$V_0$ (counts per minute in the presence of wild-type kinase) (8).

**Phospho-Acceptor Specificity of the Chimeric Kinases.**

The SH2 domain is predominantly responsible for the identification of the cellular targets of cytoplasmic tyrosine kinases (49). However, the catalytic SH1 domain does exhibit a weak peptide selectivity profile, and c-Abl and v-Src have been shown to prefer distinct optimal peptide substrates (43). The optimal peptide substrates for v-Src and c-Abl are EIQYGEF and EAIYAAPF, respectively. We asked whether protein tyrosine kinase chimeras containing the SH1 domain N-terminal lobe of c-Abl and the C-terminal lobe of v-Src preferentially phosphorylate c-Abl-like (in the presence of mutant kinase)/$V_0$ (counts per minute in the presence of wild-type kinase) (8).

**Table 3: Peptide Specificity Profiles for the Wild-Type, Engineered, and Chimeric Kinases**

<table>
<thead>
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<th>Peptide</th>
<th>v-Src</th>
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<th>c-Abl (T315A)</th>
<th>SA</th>
<th>ASA</th>
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<td>246 200</td>
<td>294 715</td>
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<td>621 400</td>
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*Counts per minute indicate the relative amounts of phosphopeptide produced upon reaction with a given kinase and [γ-32P]ATP. Kinase reactions were performed as described in Materials and Methods and were carried out for 1 h, and then the mixtures were spotted onto a phosphocellulose disk, washed, and counted for radioactivity. The concentration of each peptide substrate was 200 μM. The reaction concentration of the enzyme, wild-type or chimera, was 1–2 nM. Wild-type v-Src (XD4) and wild-type c-Abl exhibit unique peptide substrate preferences. The peptide specificity of the SA and ASA chimeras is similar to that of wild-type c-Abl and not to that of wild-type v-Src.

peptide substrates is maintained in the SA and ASA chimeric kinases. In particular, the preference for the amino acid three residues away from the phosphorylated tyrosine (the Y + 3 position) differs between c-Abl and v-Src. At this position, v-Src prefers a phenylalanine while c-Abl prefers a proline (43, 44). The SA and ASA chimeras studied here show c-Abl’s characteristic preference for proline. The v-Src and c-Abl wild-type kinases also differ in their substrate specificity with regard to the necessity for additional amino acid residues N-terminal to the phosphorylated tyrosine. v-Src and other Src family kinases phosphorylate substrates which contain only one residue, an isoleucine (such as in EIQYGEF), on the N-terminal side of tyrosine, and the addition of other residues, such as glutamate, provides only modest improvements in kinetic values (Tables 2 and 3). On the other hand, c-Abl does not phosphorylate truncated versions of its optimal substrate to as high a degree as v-Src does. It appears that c-Abl depends more upon the peptide binding contacts N-terminal to tyrosine, and a longer peptide chain of at least EIQYGEF and preferably EAIYAAPF is necessary for substantial c-Abl substrate phosphorylation. The chimeric kinases, SA and ASA, behave like wild-type c-Abl in their preference for substrates with additional N-terminal residues. Thus, the SA and ASA chimeras display a peptide specificity profile that is c-Abl-like, rather than v-Src-like as shown in Table 3. These peptide specificity data confirm that the C-terminal lobe of a tyrosine kinase catalytic domain determines peptide specificity.

**Nucleotide Specificity of the ASA and SA Chimeric Kinases.**

The I338G mutant of v-Src exhibits remarkable specificity for unnatural nucleotide analogues containing N6 substituents (4, 5). These ATP analogues are not accepted as substrates of wild-type kinases (i.e., orthogonal to the natural substrate ATP). The unnatural nucleotide specificity of v-Src (I338A) enables the direct substrates of v-Src to be identified by using [γ-32P]-N6-(benzyl)-ATP (K. Shah and K. M. Shokat, unpublished results).

The poor catalytic efficiency of the corresponding c-Abl (T315A) mutant with N6-(benzyl)-ATP (1) (Table 1 and Figure 6) precludes the use of this mutant/orthogonal nucleotide pair for tracing c-Abl protein substrates. By swapping the SH1 domain N-terminal lobe of c-Abl with that of v-Src, we hoped to create a chimeric kinase that displays the desired orthogonal nucleotide specificity of v-Src.
ASA chimera by c-Abl (T315A) (Figure 6B). The level of inhibition with the analogues (1), and (2)
killed efficiency (acceptance) of the kinase chimeras, ASA and SA, with ATP assay conditions. More importantly, the catalytic efficiency is higher than with c-Abl (T315A), respectively, under the same V
and PP1-Derivative of the same kinase. However, under the same assay conditions, the catalytic efficiency of wild-type c-Abl with ATP (Table 1).

This satisfies our design goal of engineering a kinase to use an ATP analogue with high efficiency to tag the direct donor substrates, we asked whether kinase inhibitors that bind to the N6 amine of ATP is bold. The A*TPs are

(Figure 6A) was improved over that for c-Abl (T315A) (Figure 6B). The level of inhibition with the ASA chimera with N6-(benzyl)-ATP analogues (1–3) (Figure 6A) yet retains the phospho-acceptor specificity of c-Abl. The inhibition of the ASA chimera with N6-substituted ATP analogues (1–3) (Figure 6A) was improved over that for c-Abl (T315A) (Figure 6B). The level of inhibition with the ASA chimera by N6-(benzyl)-ATP (1), N6-(2-phenethyl)-ATP (2), and N6-(3-methylbenzyl)-ATP (3) was 17, 20, and 38% higher than with c-Abl (T315A), respectively, under the same assay conditions. More importantly, the catalytic efficiency of the kinase chimeras, ASA and SA, with ATP analogues was dramatically improved. Both the ASA and SA chimera accept N6-(benzyl)-ATP with approximately 10-fold higher efficiency (kcat/KM) than ATP. The catalytic efficiency of these chimeras with N6-(benzyl)-ATP is comparable to the catalytic efficiency of wild-type c-Abl with ATP (Table 1). This satisfies our design goal of engineering a kinase to use an ATP analogue with high efficiency to tag the direct substrates of that kinase.

Inhibition of the SA and ASA Chimeric Kinases by PP1 and PP1-Derivatized Inhibitors. In addition to phospho-donor substrates, we asked whether kinase inhibitors that distinguish between v-Src and c-Abl would bind to the chimeric kinase as a means of further probing the active site formed by joining v-Src and c-Abl. The I338 residue in v-Src controls the sensitivity of inhibition of the protein kinase by a known inhibitor, 4-amino-1-tert-butyl-3-phenylpyrazolo-[3,4-d]pyrimidine (PP1) (6–8). We synthesized PP1 analogues (4–6) that can selectively inhibit the v-Src (I338A/G) mutants (Table 4) (6, 7). PP1 and its analogues can also inhibit the c-Abl (T315A) mutant but at IC50 values significantly higher than that of v-Src (I338A). PP1 is an ATP competitive inhibitor, so we asked whether the SA and ASA chimeric kinases would be potently inhibited by the same inhibitors that potently inhibited v-Src (I338A). We measured the IC50 values of the SA and ASA chimeras with PP1 and PP1 analogues. The SA and ASA chimeras were both more potently inhibited by PP1 and PP1 analogues than the c-Abl (T315A) single mutant (Table 4). Analogous to the aforementioned A*TP inhibition values, the inhibition values of the SA and ASA chimeras by PP1 analogues also fall between those of c-Abl (T315A) and v-Src (I338A). PP1 inhibits v-Src (I338A) with an IC50 of 5 nM; it inhibits c-Abl (T315A) with an IC50 of 30 nM, and it inhibits ASA with an IC50 of 15 nM. The SA and ASA chimeras gave identical IC50 values with PP1 analogue 4 (4 nM), while PP1 analogue 5 gave an IC50 of 5 nM with the ASA chimera and 6 nM with the SA chimera. These values fall between those of the v-Src (I338A) mutant (1.5 nM) for both 4 and 5 and those of the c-Abl (T315A) mutant (10 nM for both 4 and 5). PP1 analogue 6 inhibits v-Src (I338A) with an IC50 of 140 nM, but it inhibits c-Abl (T315A) to an even lesser extent (IC50 = 600 nM). However, PP1 analogue 6 inhibits the SA chimera with an IC50 of 125 nm and the ASA chimera with an IC50 of 150 nM. As predicted on the basis of the nucleotide specificity, the inhibitory values determined for the SA and ASA chimeric kinases more closely resemble those of v-Src (I338A) than those of c-Abl (T315A) as shown in Table 4.

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*IC50 is defined as the concentration of the inhibitor at which the counts per minute was 50% of the control (no inhibitor). Inhibition assays were carried out as described in Materials and Methods. The optimal peptide substrates (200 μM) were used (well above the Km value for these substrates; the Km for EIYGEFKKK with v-Src is 33–36 μM, and the Km for the Abl optimal substrate EAIYAAPFKKK is 16-20 μM for wild-type c-Abl; refer to Table 2 for peptide kinetic data). Inhibition assays were carried out for 3 h, and a 25 μL aliquot was spotted onto a phosphocellulose disk, washed, and counted for radioactivity as described.
CONCLUSION

We attempted to use the successful engineering of v-Src [v-Src (I338A)] as a blueprint to engineer unnatural nucleotide specificity into c-Abl. However, the corresponding point mutation in c-Abl (T315A) did not produce the desired property of highly efficient phosphorylation activity with the orthogonal ATP analogue N\(^6\)-(benzyl)-ATP. Attempts to identify second site revertants to c-Abl (T315A) that had the desired catalytic properties failed. Since the space-creating mutation to v-Src was successful, we decided to graft a segment of the v-Src kinase domain into c-Abl’s kinase domain as a means of enlarging the space around the N\(^6\) amino group of ATP, without compromising the catalytic activity of the kinase. Two unique chimeric kinases, SA and ASA, were constructed by swapping the SH1 domain N-terminal lobe of c-Abl with that of v-Src. Crystal structures and sequence alignments of protein kinase catalytic domains were used as a guide in the protein engineering design. The SH1 domain C-terminal lobe of c-Abl is thought to control c-Abl’s substrate specificity, hence, this protein region was preserved in the kinase chimeras. These chimeras can use an ATP analogue, N\(^6\)-(benzyl)-ATP, with high catalytic efficiency, while retaining the peptide specificity of c-Abl. Chimeric kinase domains provide a new strategy for engineering unnatural nucleotide specificity into a kinase. Due to the large number of enzymes in the protein kinase superfamily, a variety of engineering strategies will be necessary to deal with the divergent kinases that may not be amenable to a single amino acid modification strategy for altering specificity. In an attempt to utilize chemical genetic methods to alter substrate specificity for the purpose of tracing kinase signaling cascades, it is important to develop diverse engineering strategies. The highly conserved fold of the kinase domain allows multiple strategies to be used.

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REFERENCES