Engineering a "Methionine Clamp" into Src Family Kinases Enhances Specificity toward Unnatural ATP Analogues†

Scott M. Ulrich,[‡,||] Denise M. Kenski,[§,||] and Kevan M. Shokat*[,‡,⊥]

Graduate Program in Chemistry and Chemical Biology, University of California, San Francisco, California 94107-2280, Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94107-2280, and Department of Chemistry, University of California, Berkeley, California 94720

Received February 18, 2003; Revised Manuscript Received April 10, 2003

ABSTRACT: A single alanine or glycine mutation in the ATP binding site of a protein kinase allows unique use of an unnatural analogue of ATP (N^6-(benzyl) ATP) as a phosphodonor, which is not accepted by wild-type kinases. Addition of [γ^{32}P] N^6-(benzyl) ATP to a cell lysate containing an ATP analog-specific kinase allele (*as1* allele) results in the exclusive radiolabeling of bona fide substrates of the mutant kinase. Here we report efforts to engineer kinase alleles that have enhanced selectivity for ATP analogues and decreased catalytic activity with ATP, thus increasing the signal-to-noise ratio of substrate labeling. Two conserved leucine residues that contact each face of the adenine ring of ATP were mutated to methionine. The introduction of this "methionine clamp" resulted in Src and Fyn kinase alleles that have markedly improved specificity for unnatural N^6-substituted ATP analogues over the natural substrate, ATP. This preference for unnatural nucleotides is reflected in more efficient labeling of protein substrates in cell extracts using the new analogue-specific v-Src allele. Kinase alleles with enhanced selectivity for unnatural ATP analogues should greatly facilitate the ultimate goal of labeling kinase substrates in intact cells, where concentrations of ATP and other competing nucleotides are high.

Eukaryotic protein kinases are a superfamily of enzymes that catalyze phosphoryl group transfer from ATP[1] to tyrosine, serine, or threonine residues of cellular proteins. Posttranslational phosphorylation of proteins is a reversible "switch" that controls virtually every cellular process, including changes in cell morphology, progression through the cell cycle, cellular differentiation, and many others (*1*−*3*). The ubiquitous nature of protein phosphorylation is evident from the size of the kinase superfamily (575 human genes encode protein kinases (*4*)) as well as the number of cellular proteins modified by phosphorylation (∼1/3 of the expressed genome is modified by phosphorylation (*3*)). Identifying the protein substrates of each kinase is of central importance to understanding the cellular processes controlled by kinase signaling. However, due to the size of the kinase superfamily and the vast extent of cellular protein phosphorylation, the direct substrates of most protein kinases have remained elusive (*5*).

We have described a combined chemical/genetic approach that addresses the problem of identifying bona fide kinase substrates (*5*−*12*). This strategy involves introducing a functionally silent point mutation in the ATP binding site of a kinase that allows the mutant kinase to use an analogue of ATP, N^6-(benzyl) ATP, that is a poor substrate of all other protein kinases in the cell. Adding [γ^{32}P] N^6-(benzyl) ATP to a cell extract containing an N^6-(benzyl) ATP-specific kinase results in the exclusive radiolabeling of the mutant kinase's direct substrates (*5*−*7*, *9*, *10*). The mutation responsible for conferring ATP analogue sensitivity is I338G in v-Src[1], the kinase we have used as a model for protein engineering. The I338 residue contacts the N^6-position of ATP, and its truncation to alanine or glycine allows the active site to accommodate N^6-substituted ATP analogues (*13*). Sequence analysis of the kinase family has shown this residue to be a conserved, bulky hydrophobic amino acid; no human, mouse, fly, yeast, or worm protein kinase has alanine or glycine at this position (*14*). The effect of mutating this residue to alanine or glycine to generate ATP analogue-specific kinase alleles (*as1* alleles) has proven to be applicable to most protein kinases tested to date, creating the possibility of identifying the substrates of any protein kinase (*6*, *7*, *9*, *10*, *15*).

Our long-term goal is to label the direct substrates of any kinase in intact cells. In cells, kinases are localized to specialized microenvironments such as the nucleolus, chromosomes, or golgi and often participate in large (MDa) protein complexes with their substrates (*2*). These features are required for proper kinase signaling, and are difficult to recreate after solubilization of the cell membrane. Unfortunately, performing kinase substrate labeling experiments in intact cells presents significant experimental difficulties. The

† This work was supported by NIH Grant (CA70331), NSF Grant (MCB-9874587), and the Sandler Foundation.

* Corresponding author: Phone: 415-514-0472; e-mail: shokat@cmp.ucsf.edu.

|| These authors contributed equally to this work.

§ Graduate Program in Chemistry and Chemical Biology, University of California, San Francisco.

‡ Department of Cellular and Molecular Pharmacology, University of California, San Francisco.

⊥ Department of Chemistry, University of California, Berkeley.

[1] Abbreviations: v-Src, Rous sarcoma virus tyrosine kinase; GFP, green fluorescent protein; BSA, bovine serum albumin; ATP, adenosine triphosphate; GTP, guanosine triphosphate; as1, analog-sensitive; DMEM, Dubellco's Modified Eagle medium.

10.1021/bi030042a CCC: $25.00
Published on Web 06/10/2003

Table 1: Kinetic Constants for v-Src, v-Src-as1, and v-Src-as4 with ATP and N^6-(phenethyl) ATP

	ATP			N^6-(phenethyl) ATP		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,min^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,min^{-1}$)
v-Src	12 ± 3	2.0 ± 0.5	16×10^4			
v-Src-as1	148 ± 10	13 ± 1	8.7×10^4	10.0 ± 0.6	0.30 ± 0.01	3.0×10^4
v-Src-as4	208 ± 14	3.0 ± 0.1	1.4×10^4	10.0 ± 0.5	0.90 ± 0.04	9.0×10^4

first is that ATP analogues do not cross the cell membrane, and techniques that transiently permeablize the cell membrane allow only limited quantities of nucleotides to enter the cell (*16*). Second, the cellular concentration of ATP is high (1−5 mM) (*15*); thus, ATP competes with [γ^{32}P] N^6-(benzyl) ATP for the analogue-specific kinase active site, which accepts both ATP and N^6-substituted ATP analogues. Together, these problems make it difficult to identify any low abundance substrate proteins using current analogue-specific kinase alleles.

To facilitate labeling of protein kinase substrates using ATP analogues and analogue-specific kinase alleles in vivo, we have re-engineered the analogue-specific I338G v-Src allele (v-Src-as1) to (i) improve its catalytic efficiency with N^6-substituted ATP analogues, and (ii) to suppress its catalytic efficiency with ATP. Thus, efficient labeling of even low abundance substrates could occur in the presence of high ATP concentrations with limited amounts of [γ^{32}P]-labeled ATP analogue. This report describes the design and characterization of a new analogue-specific kinase allele that has dramatically increased selectivity for N^6-substituted ATP analogues over ATP.

v-Src-as1 has altered nucleotide specificity, accepting N^6-substituted ATP analogues nearly as well as ATP, with respect to k_{cat}/K_m (Table 1) (*8*). This mutation was designed to be functionally silent (remain active with ATP, and have unchanged phosphoacceptor specificity (*11*)), yet allow for a unique enzymatic activity with unnatural substrates. Engineering kinase alleles that *solely* use ATP analogues represents a quite different challenge, since a negative interaction with the natural ligand (ATP) must be introduced. A recent review article on redesigning enzyme−ligand specificity outlines several strategies for accomplishing this goal (*18*). An attractive option is to engineer an ion−pair interaction between the enzyme and an unnatural substrate, that is unsatisfied (and therefore repulsive) when the natural ligand is bound (*19*). Alternatively, engineering hydrogen bonding patterns that are unique for one substrate have been successful in converting GTPases to XTPases by a single Asp to Asn mutation (*20*). However, such options for protein kinases are limited (*12*). The only hydrogen bonds present in the kinase/ATP complex involve the protein backbone, and therefore are not easily altered by rational mutations (*21*). Also, ion−pair interactions between cationic nucleotides generated by alkylating N^1 of the purine ring cannot be matched with mutations to acidic residues, as this position is involved in one such hydrogen bond to the amide backbone. Instead, we chose to rely on further manipulation of van der Waals interactions, and engineered mutations in the kinase that would redirect the main nucleotide recognition element from the purine ring (common to ATP and N^6-substituted ATP analogues) to the N^6-substituent (specific to the ATP analogues).

MATERIALS AND METHODS

Site-Directed Mutagenesis. Point mutations in v-Src and Fyn were generated using the QuikChange protocol (Stratagene). Oligonucleotides used to generate the mutations were as follows: v-Src L273M: sense, CTCGAGGTGAA-G*ATG*GGGCAGGGCTGC; reverse complement, GCAGC-CCTGCCCC*CAT*CTTCACCTCGAG.

L393M: sense, GCCAACATC*ATG*GTGGGGGAG; reverse complement, CTCCCCCAC*CAT*GATGTTGGC. L273F: sense, CTCGAGGTGAAG*TTT*GGGCAGGGCTGC; reverse complement, GCAGCCCTGCCC*AAA*CTTCACCTCGAG. L393F: sense, GCCAACATC*TTT*GTGGGGGAG; reverse complement, CTCCCCCAC*AAA*GATGTTGGC. Fyn mutagenesis used similar oligonucleotides to convert leucines 273 and 393 to methionine.

Protein Expression and Purification. v-Src was expressed as a GST fusion protein from DH5α *E. coli* using the pGEX-KT vector system (Pharmacia). A single colony from freshly transformed bacteria was grown to saturation in 3 mL Luria broth supplemented with 50 μg/mL ampicillin. This culture was diluted into 250 mL Superbroth (Bio 101) containing ampicillin and grown at 37 °C until the OD_{595} reached 0.5. At this point, 25 μL of 1 M IPTG was added (0.1 mM final) and the culture was shaken at 23 °C for 18 h. The cells were then harvested by centrifugation at 4 °C. The wet cell pellet was resuspended in B-PER (Pierce) containing complete protease cocktail (Roche) and rocked at room temperature for 10 min. The lysate was clarified by centrifugation (15000*g*, 15 min, 4 °C), and the supernatant was applied to 40 mg (dry weight) of reconstituted glutathione beads (Sigma) and rocked at 4 °C for 2 h. The beads were then washed with ice cold TBS (3 × 7 mL, 4 °C, 15 min) and drained. Bound proteins were eluted with 10 mM free glutathione (Sigma) in 50 mM Tris (pH 8). The Bradford assay as well as Coomassie staining of an SDS−PAGE gel of the eluant was used to quantify the yield of full-length protein.

Western Blot Kinase Assays. Kinase assays were performed in a final volume of 30 μL containing 50 mM Tris (pH 8), 100 mM NaCl, 10 mM $MgCl_2$, 5 μg of GFP−IYGEF (*22*), 100 μM ATP or ATP analogue, and 100 ng of kinase. Reaction mixtures were incubated for 10 min at 23 °C then quenched by adding 6 μL of 6× Laemelli gel loading buffer and heating to 95 °C for 5 min. Proteins were resolved by SDS−PAGE (12% acrylamide) and transferred to nitrocellulose paper. The blot was probed with the antiphosphotyrosine antibody 4G10 (a generous gift from Brian Druker, OHSU) and GαM HRP followed by chemiluminescent substrates. Images were obtained using an Alpha Innotech Chemimager 5500 gel documentation system.

Determination of Kinetic Constants. Kinase reactions were performed in triplicate in a final volume of 30 μL containing 50 mM Tris (pH 8), 100 mM NaCl, 10 mM $MgCl_2$, 100 μM EIYGEFKKK peptide substrate (*10*) (∼30× the K_m), and

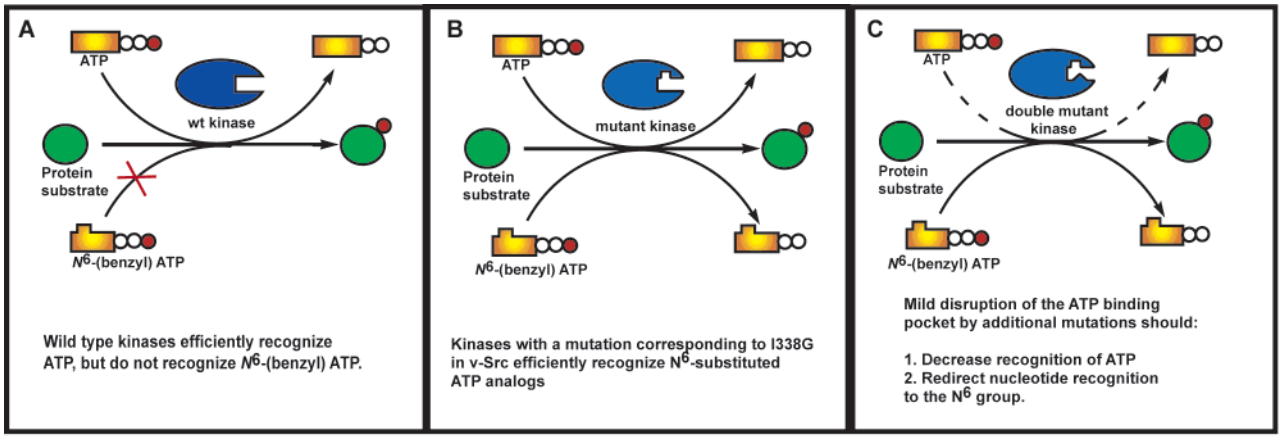

FIGURE 1: Wild-type kinases (A) cannot use N^6-substituted ATP analogues, and solely use ATP as the phosphodonor. Kinase as1 alleles (B) efficiently recognize N^6-substituted ATP analogues, as well as ATP. Design strategy for an ATP-insensitive kinase allele by selective disruption of adenine binding (C).

varying amounts of ATP or ATP analogues (New England Nuclear) with a specific activity of 5.0×10^{12} cpm/mmol. Kinase reactions were carried out for 15 min at 23 °C, then quenched by spotting 25 μL of the reaction volume on P81 phosphocellulose disks (Whatman). The disks were washed with 10% acetic acid (20 min) followed by 3×5 min washes with 0.5% phosphoric acid and a final acetone wash. Disks were then subjected to scintillation counting in 7 mL of scintillant (Scintisafe, Fischer). Data were analyzed with the Graphpad software package (Prism).

Cell Culture, Transfection, and Retroviral Infection. NIH 3T3 cells were routinely grown in DMEM with 10% donor calf serum. Wild-type and I338G v-Src (v-Src-as1) NIH 3T3 cells were routinely grown in DMEM with 10% bovine calf serum and 2.5 μg/mL puromycin (Sigma). The v-Src, v-Src-as1, and v-Src-as4 pBabe puro plasmids (gifts from Alex Muller and H. Land) were transiently transfected into Phoenix cells by the calcium phosphate transfection method. Culture medium containing the retroviruses was harvested 72 h after transfection. Infection was carried out by adding the retrovirus supernatant (3 mL) in the presence of Polybrene (4 μg/mL). After 24 h, the viral supernatant was removed and replaced with fresh media (10% donor calf serum/DMEM). After 48 h, the infected cells were selected in the presence of 2.5 μg/mL of puromycin. The expression of v-Src, v-Src-as1, and v-Src-as4 in NIH 3T3 cells was confirmed by immunoprecipitation and immunoblotting. Wild-type, v-Src-as1, v-Src-as4 proteins were present in equal amounts (data not shown).

Immunoblotting. Cells were lysed in modified RIPA buffer (1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA) and cleared by centrifugation. Cell lysates were boiled for 5 min in sample buffer and separated by 8−16% SDS−PAGE gel. Proteins were electrophoretically transferred to nitrocellulose and immunoblotted with 4G10 phosphotyrosine antibody. Immunoreactive proteins were analyzed by enhanced chemiluminescence (Pierce).

Immunprecipitaion and Kinase Assays of v-Src, v-Src-as1, and v-Src-as-4 NIH 3T3 Cells. Immunoprecipitation and kinase assays were done as described (*9*). Cells were lysed in modified RIPA buffer (1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA) with protease inhibitor cocktail (Roche, cat. 1873580), 0.1% SDS, and 2 mM sodium orthovanadate. After centrifugation at 13 000 rpm for 15 min at 4 °C, the cleared lysate was incubated with 1.5 μg of anti-v-Src antibody 327 at 4 °C for 2 h with gentle rotation, followed by incubation for 1 h with rabbit IgG (Jackson), followed by addition of 50 μL of 50% protein A Sepharose slurry for 1 h. The immune complexes were washed 3 times (500 μL each) in modified RIPA buffer without SDS and twice in kinase buffer (30 mM Tris, pH7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$). Kinase reactions were initiated by the addition of 0.5 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) alone or 1 μCi [γ-^{32}P] N^6-(benzyl) ATP (3000 Ci/mmol) in the presence of 10 μM ATP and were incubated for 10 min at room temperature. After boiling for 5 min in sample buffer, proteins were separated by 8−16% SDS−PAGE and exposed for autoradiography.

RESULTS AND DISCUSSION

To generate kinase alleles that are insensitive to ATP yet efficiently recognize N^6-substituted ATP analogues, we made specific point mutations that preserve recognition of the N^6-substituent recognition but disrupt binding to the adenine ring. I338G v-Src and N^6-substituted ATP analogues have an additional binding interaction (relative to wild-type v-Src and ATP) between the engineered I338G sub-pocket and the N^6-substituent. We viewed the adenine ring as a necessary binding element for ATP recognition, but not for recognition of N^6-substituted ATP analogues due to this additional interaction. Selectively destabilizing adenine binding should redirect the primary nucleotide recognition element to the N^6-substituent on our unnatural ATP analogues, creating a kinase allele that preferentially uses N^6-substituted ATP analogues over ATP (Figure 1C).

To test this idea, we focused on two residues that contact adenine, leucine 273, and leucine 393 of v-Src (Figure 2). Both are hydrophobic residues, allowing us to "tune" the adenine interaction by incrementally increasing the size of the residue to larger hydrophobic side chains without disrupting specific, directional contacts that may alter the nucleotide binding orientation. Both residues are conserved across the kinase family (Figure 2B), suggesting that mutations to larger hydrophobic residues may be detrimental to catalysis with ATP, the natural phosphodonor.

Leucines 273 and 393 were individually mutated to phenylalanine and methionine in the v-Src-as1 (I338G)

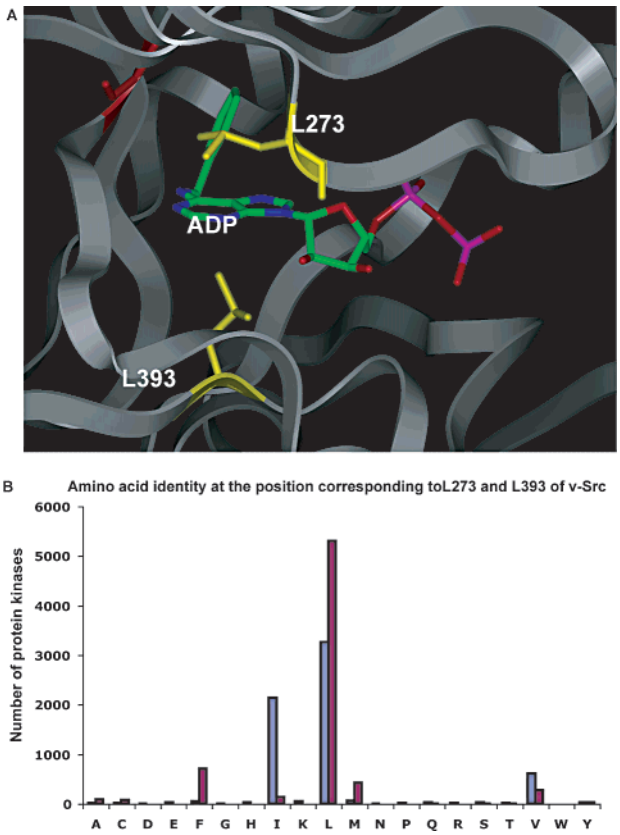

FIGURE 2: (A) Ribbon diagram from the crystal structure of v-Src-as1 with N^6-(benzyl) ATP bound (*16*). In yellow are the residues on which we focused our mutagenesis efforts: L273 (above the plane of the adenine ring) and L393 (below the plane). (B) Plot of the frequency of amino acids occurring at positions corresponding to 273 (purple) and 393 (maroon) in v-Src from a sequence analysis of the protein kinase superfamily across all organisms.

kinase, and the resulting mutants were tested for catalytic activity with ATP and three N^6-substituted ATP analogues: N^6-benzyl, -phenethyl, and -cyclopentyl ATP. The activity profiles of these mutants were compared with wt v-Src and v-Src-as1 (I338G) with the same set of nucleotides and under identical conditions.

The results of these experiments indicate that wild type v-Src does not efficiently use N^6-substituted ATP analogues, consistent with our earlier observations (*5*, *8*) that these nucleotides are orthogonal to, or poor substrates of, wild type kinases (Figure 3A, row 1). Also consistent with our earlier observations, the single I338G mutation gives v-Src-as1 the ability to efficiently use N^6-substituted ATP analogues (Figure 3A, row 2). v-Src L273F/I338G and I338G/L393F both show no detectable kinase activity with any nucleotide tested under our reaction conditions, despite repeated attempts to generate active enzyme (Figure 3A, rows 3 and 4). v-Src L273M/I338G is active and exhibits nucleotide specificity very similar to v-Src-as1, suggesting that position 273 is tolerant to conservative mutations (Figure 3A, row 5).

The v-Src I338G/L393M mutant, however, does exhibit altered nucleotide specificity. We have observed previously that v-Src-as1 (I338G) prefers ATP to N^6-(benzyl) ATP by a factor of 4 based on k_{cat}/K_m (Table 1) (*8*). However, compared to v-Src-as1 (I338G), v-Src I338G/L393M appears to slightly prefer N^6-(benzyl) ATP over ATP based on the amount to phosphoprotein detected (Figure 3A, row 6). This modest increase in catalytic selectivity led us to test the effect of mutating both leucine 273 and 393 to methionine. The resulting v-Src L273M/I338G/L393M mutant strongly prefers all N^6-substituted ATP analogues tested over ATP, and is significantly more selective for ATP analogues than v-Src-as1 (Figure 3A, row 7 vs 2). The v-Src L273M/I338G/L393M mutant is designated hereafter as *v-Src-as4*.

Next, we tested whether the two leucine to methionine substitutions at positions 273 and 393 in v-Src-as1 have the same effect on the nucleotide specificity of other Src family kinases. Fyn is a Src family tyrosine kinase central in T-cell signaling (*22*). The *Fyn-as4* (T339G/L274M/L394M Fyn) construct was generated, and the protein product was tested against the same panel of nucleotides and under the same conditions as the v-Src experiments described above. Wild-type Fyn also does not accept N^6-substituted ATP analogues (Figure 3B, row 1). Similar to v-Src-as1, Fyn-as1 (T339G) has the ability to use N^6-substituted ATP analogues (Figure 3B, row 2). Fyn-as4 had a more dramatic effect on nucleotide specificity than with v-Src-as4; no phosphoprotein product is detectable when ATP is the phosphodonor under these reaction conditions (Figure 3B, row 3).

The Enhanced Specificity for N^6-Substituted Nucleotides Is Mediated through k_{cat}, not K_m. To determine how the alterations to the adenine binding pocket in v-Src-as4 and Fyn-as4 affect nucleotide specificity, we determined the kinetic constants, K_m and k_{cat}, of v-Src-as1 and v-Src-as4 with ATP and N^6-(phenethyl) ATP (Table 1). Surprisingly, altering the adenine binding pocket of v-Src-as1 has little effect on the K_m of ATP (208 μM) or N^6-(phenethyl) ATP (10 μM). The K_m of each nucleotide is relatively unchanged when the leucine to methionine substitutions are introduced (Table 1). However, altering the adenine binding pocket does have an effect on k_{cat}; v-Src-as4 has a 4-fold lower k_{cat} of 3 min^{-1} with ATP than v-Src-as1 with a k_{cat} of 13 min^{-1}. Conversely, v-Src-as4 has a 3-fold higher k_{cat} with N^6-(phenethyl) ATP of 0.9 min^{-1} than v-Src-as1 of 0.3 min^{-1}.

Thus, targeted disruption of adenine binding has served a dual purpose. It has diminished catalytic activity with ATP, which presumably requires optimal adenine contacts. Additionally, altering the adenine binding pocket actually *improves* catalytic activity with N^6-substituted ATP analogues. Both effects sum to produce kinase alleles that are markedly more selective toward unnatural ATP molecules.

The relevant parameter for comparing competing substrates for an enzyme is the k_{cat}/K_m of each substrate. By this measure, v-Src-as1 has a 3-fold preference for ATP over N^6-(phenyl) ATP. Modifying the adenine binding pocket through the leucine to methionine substitutions inverts the nucleotide specificity such that v-Src-as4 has a 6.5-fold preference for N^6-(phenethyl) ATP over ATP.

Specificity of v-Src-as4 Is not Altered in Vivo. A key requirement of an ATP analogue specific kinase allele is that it retains the phosphoacceptor specificity of the wild-type enzyme. This feature of v-Src-as1 has been rigorously tested by a combination of an in vivo whole cell labeling, X-ray structure determination, and combinatorial peptide specificity (*8*−*13*). To also assess the phosphoacceptor specificity of v-Src-as4 we expressed the triply mutated enzyme in NIH3T3 cells. On the basis of the inverted nucleotide specificity of v-Src-as1 compared to WT and as1 alleles, we anticipated the as4 would be a less potent transforming kinase, as judged by growth in soft-agar and whole cell phosphotyrosine levels

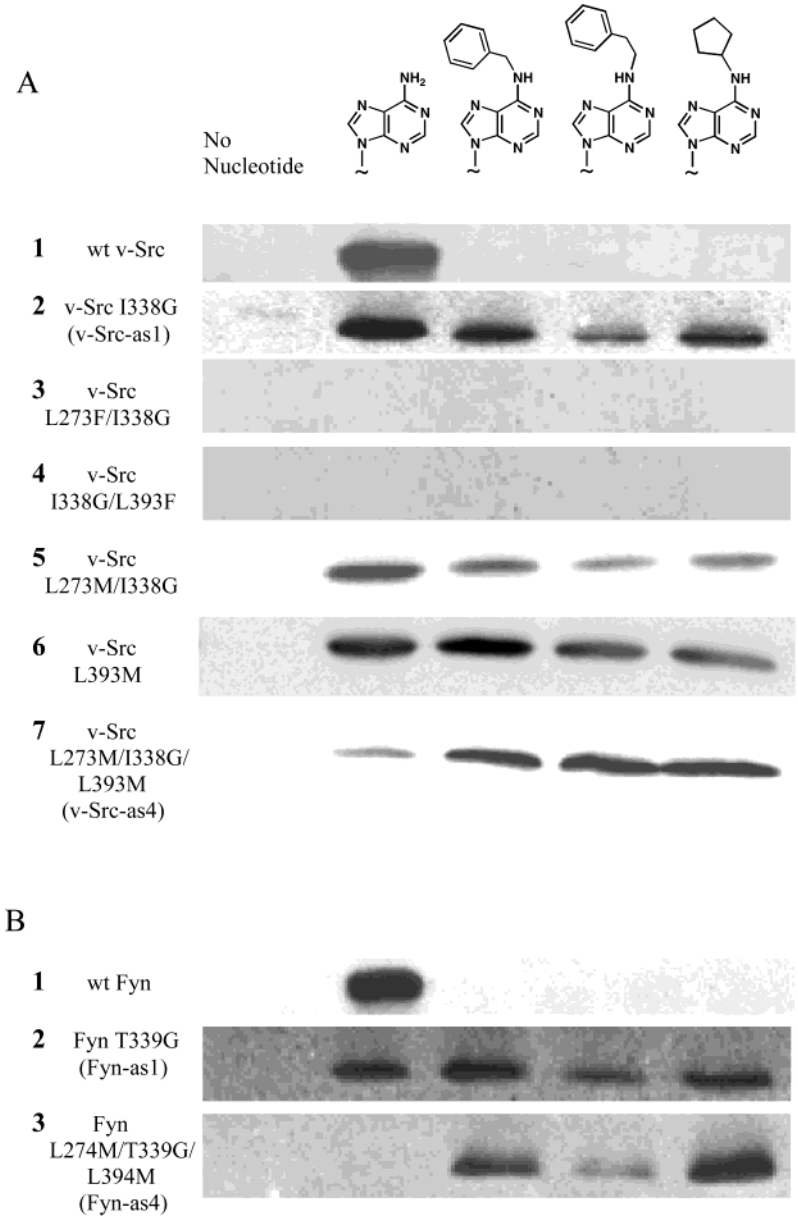

FIGURE 3: Antiphosphotyrosine Western blot of wt v-Src, v-Src-as1, and v-Src-as4. Phosphodonors correspond to no nucleotide (lane 1), ATP (lane 2), N^6-(benzyl) ATP (lane 3), N^6-(phenylethyl) ATP (lane 4), and N^6-(cyclopentyl) ATP (lane 5) (all nucleotides 100 μM). A GFP-tagged peptide substrate specific for v-Src was used as the phosphoacceptor. Row 1: Wild-type v-Src cannot use N^6-substituted ATP analogues, consistent with our earlier observations. Row 2: The I338G mutation alters the nucleotide specificity of v-Src to include N^6-substituted ATP analogues. Rows 3 and 4: Substituting phenylalanine for either leucine 273 or leucine 393 in v-Src I338G yields kinases that are inactive under any of these conditions. Rows 5 and 6: v-Src L273M/I338G and v-Src I338G/L393M have nearly identical nucleotide selectivity as v-Src I338G. Row 7: v-Src L273M/I338G/L393M has dramatically enhanced selectivity for N^6-substituted ATP analogues relative to v-Src I338G. (B) Antiphosphotyrosine Western blot of wt Fyn, Fyn-as1, and Fyn-as4 under identical reaction conditions as those in Figure 6. Row 1: Wild-type Fyn cannot utilize N^6-substituted ATP analogues. Row 2: Fyn-as1, similar to v-Src-as1, acquires the ability to use N^6-substituted ATP analogues. Row 3: Fyn-as4, like v-Src-as4, shows enhanced specificity for N^6-substituted ATP analogues, and no catalysis with ATP as the phosphodonor under these reaction conditions.

(*23*). v-Src-induced transformation of fibroblasts leads to the tyrosine phosphorylation of more than 50 proteins (*24*). Since untransformed NIH3T3 cells inhibit cellular phosphotyrosine staining, any phosphotyrosine containing proteins in v-Src wt, as1, or as4 cells can be largely, though not conclusively, attributed to phosphorylation by v-Src (*24*). Thus, the latter assay serves as a sensitive test of v-Src phosphoacceptor specificity, while soft-agar transformation is a test of the cellular consequence of the substrate protein phosphorylation.

Cells expressing v-Src, v-Src-as1, and v-Src-as4 all show significantly enhanced phosphotyrosine expression over control 3T3 cells (Figure 4). These data also indicate that wild type v-Src and v-Src-as1 cells show the same level of tyrosine phosphorylated proteins while v-Src-as4 cells had a lower response, probably due to reduced catalytic efficiency of v-Src-as4 with ATP. Nonetheless, these data show that v-Src-as1 and v-Src-as4 transformed cells are able to induce the same spectrum of tyrosine phosphorylated proteins as wild-type v-Src in vivo. Some proteins show lower levels of tyrosine phosphorylation in v-Src-as1 and v-Src-as4 transformed cell lines, while other bands remain constant across all three cells lines. This may be the result of some phosphotyrosine proteins being indirect v-Src targets and thus regulated by factors dependent upon cell state such as transformation.

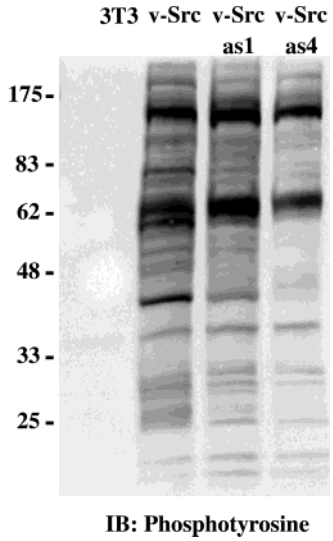

FIGURE 4: Antiphosphotyrosine Western blot of cell lysates expressing v-Src, v-Src-as1, and v-Src-as4. v-Src, v-Src-as1, and v-Src-as4 all have the same phosphotyrosine proteins. v-Src-as1and v-Src-as4 show less phosphorylation probably due to their impaired kinetics with ATP.

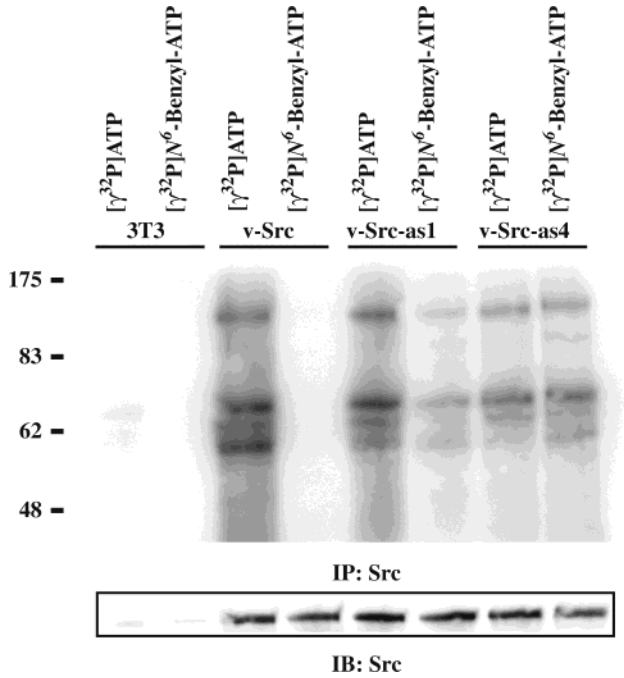

FIGURE 5: Anti-v-Src immune complex kinase reaction on v-Src, v-Src-as1, and v-Src as-4 NIH 3T3 cells. Lysates from v-Src, v-Src-as1, and v-Src as-4 NIH 3T3 cells were subjected to immunoprecipitation with anti-Src antibody. The immune complexes were incubated in the presence of [γ^{32}P] ATP or [γ^{32}P] N^6-(benzyl) ATP. While v-Src, v-Src-as1, and v-Src-as4 accept [γ^{32}P] ATP only v-Src-as1 and v-Src-as4 accept [γ^{32}P] N^6-(benzyl) ATP.

In support of this, we determined how v-Src-as4 functions in vivo. We delivered genes encoding v-Src, v-Src-as1, and v-Src-as4 into NIH3T3 fibroblasts via retroviral infection and measured the transformation efficiency, phosphotyrosine expression of resulting cell lines and kinase substrate activity of immunoprecipitated v-Src alleles. The ability of genes to transform was also measured by the soft agar assay (*23*). Both v-Src and v-Src-as1 transfection result in transformed cells, while cells expressing v-Src-as4 were not transformed, in line with the reduced catalytic efficiency of v-Src-as4 with the natural phosphodonor, ATP (data not shown).

To directly examine phosphoacceptor and nucleotide specificity of these v-Src alleles in this cellular context, we generated v-Src immunoprecipitations and treated them with radiolabeled ATP and ATP analogues (Figure 5) (*9*). Using ATP as a phosphodonor, it is again clear that v-Src-as1 and v-Src-as4 have identical protein substrate specificity as wild-type v-Src. However, the data demonstrate a marked improvement in substrate labeling due to nucleotide specificity. Wild-type v-Src does not accept N^6-(benzyl) ATP while v-Src-as1 and v-Src-as4 do, consistent with earlier data (Figure 5). Quantitation of the band intensity using densitometry shows that v-Src-as1 prefers ATP 5-fold over N^6-(benzyl) ATP, while v-Src-as4 uses N^6-(benzyl) ATP to the same extent as ATP for the protein substrates at 70 and 160 kDa, under these immunoprecipitation kinase assay conditions. This is in agreement with our kinetic measurements with peptide substrates and shows that the increased selectivity of v-Src-as4 for N^6-(benzyl) ATP does result in more robust substrate labeling. Future assessment of the true phosphoacceptor specificity of v-Src-as4 will require the use of peptide substrate libraries as well as X-ray crystallographic characterization (*15*).

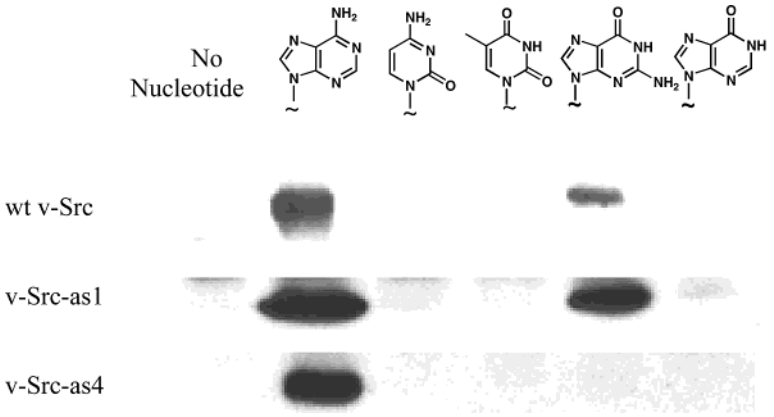

FIGURE 6: Antiphosphotyrosine Western blot of v-Src, v-Src-as1, and v-Src-as4 with a panel of alternative nucleotides as phosphodonors (100 μM) using a GFP-tagged peptide substrate specific for v-Src. Lane 1, no nucleotide; lane 2, ATP; lane 3, CTP; lane 4, TTP; lane 5, GTP; lane 6, ITP. Rows 1 and 2: v-Src and v-Src-as1 both have the ability to use GTP as an alternate phosphodonor, indicating an intact purine binding pocket. Row 3: v-Src-as4 has undetectable catalytic activity with GTP, suggesting suppressed recognition of purine nucleotides that lack N^6 substituents.

The Methionine Substitutions Suppress Catalytic Activity with other Purine Nucleotides. We have shown as4 alleles have enhanced specificity for N^6-substituted ATP analogues over ATP. However, methionine-rich binding sites are thought to be promiscuous binders, presumably due to the unusual conformational mobility and polarizibility of methionine (*26*). Also, other natural nucleotides such GTP can substitute for ATP as a phosphodonor for some kinases (*25*). Since these nucleotides all lack the interaction between the N^6-substituent and the I338G subpocket, we predict that they should have lower catalytic activity with v-Src-as4 than v-Src-as1. In addition to probing our model of the catalytic enhancement through introduction of the methionine "clamp", assaying natural nucleotide triphosphates with v-Src-as1 and v-Src-as4 would also ascertain if the methionine-rich active site of Src family as4 alleles have become promiscuous nucleotide binders.

Using prolonged reaction times (1 h), wt v-Src and v-Src-as1 are capable of using GTP as an alternate phosphodonor (Figure 6). Wild type v-Src and v-Src-as1 kinase activity supported by GTP is not as robust as that with ATP, but the guanosine scaffold is clearly tolerated. v-Src-as4, however, loses the ability to support catalysis with a nonoptimal purine nucleotide (GTP), just as catalysis with the optimal purine (ATP) is suppressed. This supports our general model that the methionine "clamp" selectively disrupts purine ring binding, thereby discriminating against nucleotides that lack N^6-substituents.

CONCLUSIONS

Our laboratory has described a method to label the bona fide substrates of protein kinases by engineering kinase alleles (*as1*) that are sensitive to N^6-substituted ATP analogues that are poor substrates for all wild-type kinases (*9*). Adding [γ^{32}P] labeled N^6-substituted ATP analogues to a cell lysate or immunoprecipitate containing a kinase-as1 allele results in specific radiolabeling of its direct substrates (*5*, *8*). Ideally, such substrate tagging experiments would be carried out in live or intact cells, where kinases and substrates are in their relevant cellular location and are involved in physiological protein−protein interactions.

In this report, we have described new Src family kinase as4 alleles, which display ~20-fold greater catalytic specificity for N^6-substituted ATP analogues than the corresponding as1 alleles. More importantly, the as4 alleles also prefer to use N^6-substituted ATP analogues over ATP both in vitro and in cultured cell lines. This preference for N^6-substituted ATP analogues is manifested through k_{cat}, where there is reduced catalytic activity with ATP while use of N^6-substituted ATP analogues is improved. The Src family kinase as4 alleles should facilitate substrate labeling experiments in vivo where the concentration of natural nucleotides is high and would inhibit the use of N^6-substituted ATP analogues by kinases, such as the as1 alleles, that also efficiently use ATP. As shown by phosphotyrosine western blots and immunoprecipitation kinase assays the as4 allele is able to be active in vivo and preferentially use N^6-(benzyl) ATP over ATP to label its substrates.

Our model explaining inverted nucleotide specificity is based on methionine side chains forming a "clamp" on each face of the purine ring of ATP, specifically disrupting purine ring binding. Since the as4 allele also has the I338G mutation it is able to accommodate the N^6-substituted ATP analogues. This selective destabilization redirects the primary nucleotide recognition element to the N^6-substituent of unnatural ATP analogues. This idea is supported by the observation that the Src-as4 allele has lower catalytic efficiency with ATP as well as the alternate purine nucleotide substrate, GTP.

This study highlights the subtle factors that must be considered when redesigning the interaction of enzymes and substrates through manipulation of van der Waals contacts between the enzyme and substrate. We believe the properties we have engineered into the Src family kinase-as4 mutants will expand the scope of using N^6-substituted ATP analogues to label direct kinase substrates of this important gene family.

ACKNOWLEDGMENT

We thank members of the Shokat lab, particularly PJ Alaimo for helpful advice and critical reading of the manuscript, and Cellular Genomics for providing radiolabeled ATP analogues. We also thank the UCSF Mass Spectrometry Facility.

REFERENCES

1. Hunter, T. (2000) *Cell 100*, 113−127.
2. Pawson, T., and Nash, P. (2000) *Genes Dev. 14*, 1027−1047.
3. Cohen, P. (2000) *Trends Biochem. Sci. 25*, 596−601.
4. Lander, E. S., et al. (2001) *Nature 409*, 860−921.
5. Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3565−3570.
6. Habelhah, H., Shah, K., Huang, L., Burlingame, A. L., Shokat, K. M., and Ronai, Z. (2001) *J. Biol. Chem. 276*, 18090−18095.
7. Habelhah, H., Shah, K., Huang, L., Ostareck-Lederer, A., Burlingame, A. L., Shokat, K. M., Hentze, M. W., and Ronai, Z. (2001) *Nat. Cell Biol. 3*, 325−330.
8. Liu, Y., Shah, K., Yang, F., Witucki, L., and Shokat, K. M. (1998) *Chem. Biol. 5*, 91−101.
9. Shah, K., and Shokat, K. M. (2002) *Chem. Biol. 9*, 35−47.
10. Polson, A. G., Huang, L., Lukac, D. M., Blethrow, J. D., Morgan, D. O., Burlingame, A. L., and Ganem, D. (2001) *J. Virol. 75*, 3175−84.
11. Witucki, L. A., Huang, X., Shah, K., Liu, Y., Kyin, S., Eck, M. J., and Shokat, K. M. (2002) *Chem. Biol. 9*, 25−33.
12. Ulrich, S. M., Buzko, O., Shah, K., and Shokat, K. M. (2000) *Tetrahedron 56*, 9495−9502.
13. Liu, Y., Shah, K., Yang, F., Witucki, L., and Shokat, K. M. (1998) *Bioorg. Med. Chem. 6*, 1219−1226.
14. Buzko, O., and Shokat, K. M. (2002) *Bioinformatics 9*, 1274−5.
15. Chaudhary, A., Brugge, J. S., and Cooper, J. A. (2002) *Biochem. Biophys. Res. Commun. 294*, 293−300.
16. Chitu, V., Demydenko, D., Toth, G. K., Hegedus, Z., and Monostori, E. (1999) *Biotechniques 27*, 435−437.
17. Voet, D., and Voet, J. (1995) *Biochemistry*, Wiley, New York.
18. Koh, J. T. (2002) *Chem. Biol. 9*, 17−23.
19. Cronin, C. N., and Kirsch, J. F. (1988) *Biochemistry 27*, 4572−4579.
20. Hwang, Y. W., and Miller, D. L. (1987) *J. Biol. Chem. 262*, 13081−13085.
21. Ting, A. Y., Witte, K., Shah, K., Kraybill, B., Shokat, K. M., and Schultz, P. G. (2001) *Biopolymers 60*, 220−8.
22. Yang, F., Liu, Y., Bixby, S. D., Friedman, J. D., and Shokat, K. M. (1999) *Anal. Biochem. 266*, 167−173.
23. Fan, Q. W., Zhang, C., Shokat, K. M., and Weiss, W. A. (2002) *Curr. Biol. 12*, 386-94.
24. Brown, M. T., and Cooper, C. J. (1996) *Biochim. Biophys. Acta 287*, 121−149.
25. Niefind, K., Putter, M., Guerra, B., Issinger, O. G., and Schomburg, D. (1999) *Nat. Struct. Biol. 6*, 1100−1103.
26. Gellman, S. H. (1991) *Biochemistry 30*, 6633−6636.

BI030042A