Precision Substrate Targeting of Protein Kinases v-Abl and c-Src*

Tae Ryong Lee‡, Jeffrey H. Till§, David S. Lawrence‡, and W. Todd Miller§

From the Departments of Chemistry and Medicinal Chemistry, Natural Sciences and Mathematics Complex, State University of New York, Buffalo, New York 14260 and the Department of Physiology and Biophysics, School of Medicine, State University of New York, Stony Brook, New York 11794

The implications of these observations are 2-fold. First, the ability of c-Src to utilize short chain alcohols as substrates, an activity virtually absent from the catalytic repertoire of v-Abl. The implications of these observations are 2-fold. First, the ability of c-Src to utilize short chain alcohols as substrates, an activity virtually absent from the catalytic repertoire of v-Abl.

Second, because these enzymes exhibit disparate active site specificities, it is possible that other tyrosine-specific protein kinases will display unique substrate specificities as well. Consequently, it may ultimately be possible to exploit these differences to generate inhibitors that precisely target specific protein kinases.

Protein phosphorylation plays a pivotal role in signaling pathways, which regulate such fundamental processes as cell growth, differentiation, and division (1, 2). Consequently, protein kinase inhibitors that can be precisely directed to specific members of the kinase family could prove to be of decided utility in understanding and controlling these signaling pathways. Much of the effort devoted to the design and construction of agents that are targeted to individual protein kinases has focused on the identification of characteristic substrate sequence specificities. Because most protein kinases utilize short peptides as substrates (3, 4), it may be possible to create inhibitors for specific protein kinases by employing peptides containing appropriate primary sequences. Unfortunately, this approach suffers from the limitation that many kinases exhibit broad overlapping sequence specificities with other family members. One way to overcome this obstacle is to employ additional recognition motifs in conjunction with the specific primary sequence to narrow the range of kinases that are impaired by inhibitory species.

We have developed a facile method to rapidly assess the active site substrate specificity of protein kinases (5–11). Although protein kinases will generally phosphorylate only serine, threonine, and/or tyrosine residues in intact protein substrates, we have found that these enzymes will phosphorylate a diverse array of alcohol-containing non-amino acid residues in synthetic peptides. This approach has allowed us to investigate the range of functionality that can be accommodated within and phosphorylated by the protein kinase active site, an issue particularly germane to the development of such inhibitory species as transition state analogs, affinity labels, and mechanism-based inhibitors. In addition, although many serine/threonine-specific protein kinases exhibit overlapping specificities for conventional peptides, we have recently shown that several of these enzymes will display remarkably different behavior toward peptides bearing exotic alcohol-containing residues (9).

In short, it may ultimately be possible to target specific members of the protein kinase family by coupling the active site substrate specificity of an individual family member with its characteristic sequence specificity.

We recently described the first active site substrate specificity analysis of a tyrosine-specific protein kinase (11). The specificity of c-Src, like that of several serine/threonine-specific protein kinases, is not limited to amino acid residues found in proteins. Indeed, c-Src phosphorylates a broad assortment of aliphatic and aromatic alcohol-containing residues that are attached to active site-directed peptides. Do members of the tyrosine-specific protein kinase family, like their serine/threonine-specific counterparts, exhibit dissimilar active site specificities? If the answer to this question is in the affirmative, it may be possible to exploit these differences in specificity to create inhibitory agents that distinguish between otherwise closely related enzymes. As a consequence, we have investigated the active site specificity of a second “tyrosine-specific” protein kinase.

The protein product of v-abl, like that of c-src, specifically phosphorylates tyrosine residues in protein substrates. The oncogenic form of abl was first discovered as the transforming gene of Abelson murine leukemia virus (12). In humans, the normal cellular homolog, c-abl, is involved in chronic myelogenous leukemias and some acute lymphocytic leukemias (reviewed in Ref. 13). In these leukemias, a chromosomal translocation takes place in which 5' sequences of the bcr gene become fused to abl, generating an oncogenic Bcr-Abl fusion protein (14). In all cases, there is a strong correlation between oncogenic transformation and tyrosine kinase activity. Like

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† To whom correspondence should be addressed. Tel.: 716-645-6800, ext. 2170; Fax: 716-645-6963.

‡ Department of Chemistry and Medicinal Chemistry, State University of New York, Buffalo, New York 14260.
§ Department of Physiology and Biophysics, School of Medicine, State University of New York, Stony Brook, New York 11794.

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aβl, src was originally identified as a retroviral oncogene; both transforming genes are altered versions of normal cellular proto-oncogenes (15). v-Abl and c-Src are members of the nonreceptor tyrosine kinase family and, as such, share many common features. Both contain SH2 and SH3 domains (16, 17), and both Src and several forms of Abl undergo myristoylation (18, 19), which promotes membrane association. These enzymes also share a strong sequence homology within their respective active site regions (20, 21). Finally, both kinases exhibit overlapping substrate specificities. A feature that can render the design of kinase-specific inhibitors problematic. In spite of these similarities, we now report that these tyrosine-specific kinases can be distinguished from one another by their active site substrate specificities.

MATERIALS AND METHODS

All chemicals were obtained from Aldrich, except for [γ-32P]ATP (DuPont NEN), piperidine (Advanced ChemTech), protected amino acid derivatives (Advanced ChemTech and Bachem), Liquiscint (National Diagnostics), Fmoc-Leu-2-methoxy-4-alkox benzyl (benzyl) alcohol resin (Peninsula), glutathione-agarose (Molecular Probes), and N-laurylsarcosine (Sigma). Phosphorallllumine P 81 paper discs were purchased from Whatman. 14 NMR and 13C NMR experiments were performed at 400 (Varian VXR-400S) and 22.5 MHz (Varian Gemini-300, respectively). Chemical shifts are reported with respect to tetramethylsilane. Fast atom bombardment (peptides) and electron impact (amino alcohols) mass spectral analyses were conducted with a VG-70SE mass spectrometer.

Amino Acid Syntheses—All amino acid derivatives were purchased from Aldrich except for 4-aminomethylbenzyl alcohol, 3-aminopropylbenzyl alcohol, 7-amino-1-heptan, 8-amino-1-octan, 10-amino-1-decan, and 12-amino-1-dodecan. The latter compounds were prepared as previously described (11).

Peptide Synthesis—Fmoc-Arg(Mtr)-Arg(Mtr)-Arg(Mtr)-Arg(Mtr)-Leu-Glu(butyl)-Glu(butyl)-Leu-Leu-resin was prepared using an automated peptide synthesizer (Advanced ChemTech Act 90). Upon completion of peptide synthesis, the side chain-protected peptide was cleaved from the resin using 1% trifluoroacetic acid in CH2Cl2 (20 ml/g resin, 15 min). The resin was filtered, and the trifluoroacetic acid/CH2Cl2 solvent (containing the peptide) was added to a 10-fold range around the apparent Km, Km). Phosphorylation reactions were initiated by the addition of 10 μM of c-Src diluted from a concentrated stock solution (6.2 nm (or where appropriate, 3.1 nm) in 1 mL diethanol and 20 mL Heps, pH 7.5) to a solution containing 15 μL of peptide (20 μM Heps, pH 7.5) and 15 μL of assay buffer (20 μM Heps, 0.33 mg/mL bovine serum albumin, 53.3 mg/mL MgCl2, and 26.67 mM Na3VO4), and 1.55 nM c-Src (except for peptides 1 and 2, where [c-Src] = 0.775 nm). For the determination of kinetic constants, the following concentrations were employed: 100 μM [γ-32P]ATP (1000–2000 cpm/pmol) and a substrate concentration over a 10-fold range around the apparent Km, Km). Phosphorylation reactions were initiated by the addition of 10 μM of c-Src diluted from a concentrated stock solution (6.2 nm (or where appropriate, 3.1 nm) in 1 mL diethanol and 20 mL Heps, pH 7.5) to a solution containing 15 μL of peptide (20 μM Heps, pH 7.5) and 15 μL of assay buffer (20 μM Heps, 0.33 mg/mL bovine serum albumin, 53.3 mg/mL MgCl2, and 26.67 mM Na3VO4). Reactions were terminated after 20.0 min by spotting 25-μL aliquots onto 2.1-cm diameter phosphocellulose paper discs. The amino acids were immersed in 10% glacial acetic acid and allowed to soak with occasional stirring for at least 1 h. The acetic acid was decanted, and the discs were collectively washed with four volumes of 0.5% H3PO4 and one volume of water followed by a final acetic rinse. The discs were air dried and placed in plastic scintillation vials containing 6 mL of Liquiscint prior to scintillation counting for radioactivity.
is clear that this peptide is more efficiently phosphorylated by
noted (15, 28).2
common substrates, a feature that has been previously
latter. The salient point is that both enzymes phosphorylate
serve as more potent phosphoryl transfer catalyst than the
substrates reported for this enzyme (15).2 In short, the fact that
comparatively charged, a feature that appears to be important for
method for following the kinetics of phosphorylation. The C-
phosphocellulose filter paper. This provides a convenient
in order to promote binding of the phosphorylated peptide to
arginine residues on the N terminus of this peptide are present
discrimination between aromatic and aliphatic alcohols (5).
However, whereas c-Src does not.
We previously demonstrated that the serine/threonine-specific
AMP-dependent protein kinase will not catalyze the phospho-
ylation of tyrosine in an active site-directed peptide (29).
However, this protein kinase will catalyze the phosphorylation of
aromatic alcohols if the hydroxyl moiety can be positioned in
the active site in a manner comparable with that of serine (8, 10).
In addition, although cAMP-dependent protein kinase (as well as protein kinase C) will phosphorylate adhial aliphatic
alcohols, this activity is highly dependent upon chain length (5, 9). There is a dramatic loss in substrate efficacy if the number
of methylene groups on the residue to be phosphorylated is
greater than two. This is not particularly surprising, because
the hydroxyl and amino groups in serine are separated by two
methylene groups as well. The active site substrate specificity of
serine/threonine-specific protein kinases is therefore strictly
determined by the relative orientation of and the distance between
the phosphorylatable alcohol moiety and the peptide backbone
to which it is attached. Is the active site specificity of tyrosine-specific protein kinases as tightly regulated? The results
in Table I clearly demonstrate that both c-Src and v-Abl will phosphorylate aromatic and benzylic (i.e. aliphatic) alcohols.
However, each of these substrates possesses an aromatic moiety.
Furthermore, the hydroxyl functionality in species 2–4 is oriented, relative to the peptide backbone, in a manner reminiscent of that found in the tyrosine-containing peptide 1.

### RESULTS AND DISCUSSION

Peptide substrates containing structurally disparate alcohol-bearing residues, positioned at the site of phosphorylation, can provide critical information concerning the range of compounds that can be accommodated within and phosphorylated by the active site of protein kinases (5). Although it can be laborious to synthesize peptides that contain exotic amino acid residues at internal sites, it requires significantly less effort to prepare analogues containing nonstandard residues at either the N or the C terminus (5). Both c-Src and v-Abl phosphorylate the C-terminal tyrosine residue in peptide 1 (Table I). The five arginine residues on the N terminus of this peptide are present in order to promote binding of the phosphorylated peptide to phosphocellulose filter paper. This provides a convenient method for following the kinetics of phosphorylation. The C-terminal portion of the peptide, Leu-Glu-Glu-Leu-Leu, is negatively charged, a feature that appears to be important for substrate recognition by some tyrosine kinases (15, 26, 27).2 However, whereas 1 is a substrate for both c-Src and v-Abl, it is clear that this peptide is more efficiently phosphorylated by the former than by the latter. This may be due, in part, to real differences in sequence specificities between the two enzymes. For example, Src has a special preference for peptide substrates that contain acidic residues at the P-3 and P-4 positions (27).2 In contrast, v-Abl is much less dependent upon the presence of negatively charged residues for substrate recognition (26, 27). However, it is also important to note that kinetic constants obtained for the v-Abl-catalyzed phosphorylation of 1 compare favorably with those associated with the best peptide substrates reported for this enzyme (15).2 In short, the fact that peptide 1 is a 42-fold more efficient substrate for c-Src than for v-Abl may simply be due to the innate ability of the former to serve as a more potent phosphotransfer catalyst than the latter. The salient point is that both enzymes phosphorylate common substrates, a feature that has been previously noted (15, 28).2

Both v-Abl and c-Src phosphorylate aromatic and benzylic alcohols (Table I). For example, peptide 2, which contains an

### Table I

| Peptide | Kinetic Constants (μM) | Vmax (nmol/min · mg) |
|---------|------------------------|----------------------|
| c-Src   | K<sub>m</sub> | V<sub>max</sub> | c-Src | K<sub>m</sub> | V<sub>max</sub> | v-Abl | K<sub>m</sub> | V<sub>max</sub> |
| 1       | 789 ± 202           | 2190 ± 325          | 524 ± 75     | 34.6 ± 2.0 |
| 2       | 396 ± 61            | 309 ± 31            | 982 ± 200    | 5.0 ± 0.6  |
| 3       | 182 ± 34            | 53 ± 3              | 764 ± 136    | 4.9 ± 0.3  |
| 4       | 645 ± 78            | 111 ± 6             | 889 ± 120    | 5.3 ± 0.2  |

Determination of Kinetic Constants—The apparent K<sub>m</sub> (± S.D.) and V<sub>max</sub> (± S.D.) values were determined from initial rate experiments. The data from these experiments were plotted using the Lineweaver-Burke procedure, and the corresponding plots proved to be linear.

### Table II

A comparison of the substrate specificity of peptides 1–19 for c-Src and v-Abl

| Peptide | c-Src K<sub>m</sub>/V<sub>max</sub> | v-Abl K<sub>m</sub>/V<sub>max</sub> | c-Src-v-Abl specificity |
|---------|-------------------------------|-------------------------------|------------------------|
| 1       | 166 ± 17                      | 3.91 ± 0.30                   | 42:1                   |
| 2       | 48 ± 3                        | 0.30 ± 0.03                   | 160:1                  |
| 3       | 17 ± 2                        | 0.39 ± 0.05                   | 42:1                   |
| 4       | 10 ± 1                        | 0.36 ± 0.04                   | 28:1                   |
| 5       | 27 ± 6                        | 0.013<sup>a</sup>             | 2076:1                 |
| 6       | 20 ± 3                        | 0.018<sup>b</sup>             | 1111:1                 |
| 7       | 19 ± 1                        | 0.023 ± 0.001                 | 826:1                  |
| 8       | 19 ± 2                        | 0.067 ± 0.003                 | 284:1                  |
| 9       | 23 ± 2                        | 0.095 ± 0.001                 | 242:1                  |
| 10      | 32 ± 1                        | 0.15 ± 0.01                   | 213:1                  |
| 11      | 35 ± 1                        | 0.25 ± 0.02                   | 140:1                  |
| 12      | 14 ± 1                        | 0.22 ± 0.02                   | 64:1                   |
| 13      | 15 ± 1                        | 0.12 ± 0.02                   | 13:1                   |
| 14      | 6 ± 1                         | 0.011<sup>c</sup>             | 545:1                  |
| 15      | 19 ± 1                        | 0.06 ± 0.01                   | 233:1                  |

<sup>a</sup>These K<sub>m</sub>/V<sub>max</sub> values were obtained using the assumption that substrate concentrations employed were less than the K<sub>m</sub>.
Is this structural alignment of functionality a requirement for recognition by tyrosine-specific protein kinases? In order to address this question, we prepared a series of peptides containing straight chain aliphatic alcohols. In a broad sense, both v-Abl and c-Src utilize these species as substrates (Table III). Therefore, it is evident that an aromatic moiety is not required for substrate recognition by either of these enzymes. Furthermore, the distance between the aromatic alcohol in tyrosine and the α-amino group is roughly equivalent to a chain containing 5-7 methylene groups. Consequently, one might predict that compounds 8-10 should serve as the most efficient substrates for v-Abl and c-Src and that the remainder of the compounds listed in Table III would be extraordinarily inefficient substrates (if they are phosphorylated at all). Somewhat surprisingly, this is not the case (Table II). For example, the octanolamine derivative 11 is the most effective c-Src substrate. However, the \( K_m / K_{cat} \) values for peptides 5-12 do not differ to any significant extent with this enzyme. Only at a chain length of 12 carbon atoms is a drop-off in substrate efficacy apparent. In this particular case, we were only able to obtain a lower limit of the maximal velocity. This is due to the fact that substrate saturation appears to occur at a relatively low concentration (<150 \( \mu M \)) and that at these concentrations the rate of phosphorylation of 13 is exceedingly weak. This implies that the concentrations of 13 employed may exceed the \( K_m \). As a consequence, we are able to provide only an estimate of \( V_{max} \) for this peptide, as well for peptides 15-18 in Table IV.

In contrast to the behavior displayed by c-Src toward peptides 5-13, v-Abl exhibits a definite dependence upon chain length. Short chains, such as the ethanolamine (5) and propanolamine (6) derivatives, are extremely poor v-Abl substrates. Interestingly, our inability to obtain \( K_m \) and \( V_{max} \) values in these two instances is not due to the difficulties that we encountered with the c-Src-catalyzed phosphorylation of peptide 13 described above. In the case of v-Abl, although high concentrations (1-2 mM) of peptides 5 and 6 provide weak rates of phosphorylation, we did not detect any evidence of substrate saturation. This implies that the \( K_m \) values for peptides 5 and 6 are quite large, a notion consistent with the results obtained for peptide 7, the next higher homolog. At substrate concentrations significantly below the \( K_m \), the Michaelis-Menten equation reduces to the initial Michaelis-Menten form, which allows the ready evaluation of the rate of phosphorylation by this enzyme as well (Table IV). However, we are uncertain of the actual \( K_m \) the \( K_{cat} / K_m \) values indicated for peptides 5, 6, and 14 in Table II should be viewed as approximations. Finally, the v-Abl-catalyzed rates of phosphorylation for peptides 15-18 are so weak that we could only provide an estimate of \( K_{cat} / K_m \) in these cases.

Based upon the \( K_{cat} / K_m \) values extracted for peptide 5, it is evident that this species is a significantly more potent substrate (>2,000-fold) for c-Src than for v-Abl. As such, peptide 5 is the most accurate c-Src-targeted substrate that we have evaluated to date. However, this bias in favor of c-Src decreases in a dramatic fashion with increasing chain length. This decrease is a consequence of the lower homolgy of the customarily used substrates. Consequently, this bias decreases as chain length increases. However, the ethanolamine-containing peptide 5 serves as a substrate for c-Src, whereas it is not too surprising that Arg-Arg-Arg-Arg-Glu-Leu-Glu-Leu-Leu-Ser-amide (14) is phosphorylated by this enzyme as well (Table IV). However, the ethanolamine derivative (5) is 4.5-fold more efficient than the serine-containing counterpart (14). One possible explanation for this observation is that the amide moiety, which is present in 14 but absent in 5, encounters unfavorable steric interactions with active site residues. If this supposition is correct, then substrates more sterically demanding than the amide functionality should produce even less effective substrates (Table IV). Indeed, this appears to be the case for the benzyl-substituted derivatives 15 and 16. These results are not particularly unexpected given the structural characteristics of the customary amino acid side chain.
substrate of c-Src. In the case of tyrosine, the hydroxyl moiety protrudes from an aromatic framework, which is tied back in a sterically uncompromising cyclic fashion. In contrast, the amide and benzyl substituents of peptides 14–16 are more sterically prominent and obtrusive than the aromatic moiety of tyrosine, particularly near the hydroxyl functionality. Furthermore, secondary alcohols (17 and 18) also fail to serve as efficient c-Src substrates. All of these observations are consistent with the notion that sterically demanding functionality may encounter unfavorable interactions when forced to reside within the active site region of c-Src. Although the ethanalamine-derivated peptide 5 is an effective c-Src substrate, it is almost imperceptibly phosphorylated by v-Abl. If the steric arguments outlined above apply to v-Abl as well, then at the very least this enzyme should not be able to phosphorylate peptides 14–18 any more efficiently than 5. Indeed this is the case. Nevertheless, v-Abl does phosphorylate all of these peptides, albeit at an nearly indiscernible level that is barely above background.

Protein kinases are commonly classified by their ability to phosphorylate aliphatic (i.e. serine/threonine) or aromatic (i.e. tyrosine) hydroxyl groups in naturally occurring proteins. However, as is apparent from this as well as earlier (5–11) active site substrate specificity studies, these categories break down with synthetic substrates. Nonetheless, a new pattern is beginning to emerge with respect to the classification of protein kinases. We, as well as others, have shown that although cAMP-dependent protein kinase will only phosphorylate \( \alpha \)-amino acid residues, protein kinase C will catalyze the phosphorylation of both \( \beta \)- and \( \delta \)-stereoisomers (5, 9, 30, 31). More recently, cGMP-dependent protein kinase and c-Src (11) have been added to the latter category. We have now found that peptide 19, which contains a \( \delta \)-tyrosine residue, will also serve as a substrate for v-Abl. The following features concerning this activity are notable. First, although v-Abl will phosphorylate peptides containing either \( \delta \)- or \( \lambda \)-tyrosine, the latter is a significantly more efficient (65-fold) substrate than the former. Second, the \( \delta \)-tyrosine-containing peptide 19 is a 5-fold poorer substrate than the corresponding achiral species (2). Consequently, it is clear that the inverted configuration associated with \( \delta \)-tyrosine in conjunction with the amide substituent actively interferes with the ability of this species to be recognized as a substrate for v-Abl. Third, peptide 19 serves as a 230-fold more efficient substrate for c-Src than for v-Abl, which may indicate that the former is able to accommodate \( \alpha \)-amino acid residues more readily than the latter.

We have not yet completely unraveled the molecular mechanism that enables certain protein kinases to phosphorylate both \( \lambda \)- and \( \delta \)-configurational isomers. However, we have previously proposed that the alcohol functionality of a \( \delta \)-residue can be presented to the target kinase in a manner analogous to that of an \( \lambda \)-residue if the peptide were to bind to the enzyme surface in a aberrant C to N terminus fashion (9). Although an analogous binding mode may be in operation for v-Abl and c-Src, other mechanisms may account for this form of active site specificity as well.

In summary, we have compared and contrasted the active site substrate specificities of v-Abl and c-Src. Although these enzymes are commonly referred to as tyrosine-specific, both species are able to phosphorylate a wide variety of aromatic and aliphatic alcohols, including a \( \delta \)-tyrosine residue. Both are also unable to phosphorylate certain structural arrangements, such as secondary alcohols and primary alcohols containing large substituents within close proximity to the hydroxyl moiety. Nevertheless, v-Abl and c-Src do exhibit certain subtle and gross differences in catalytic behavior as well. Although c-Src displays a moderate preference for aromatic versus benzylic alcohols, v-Abl does not. Indeed, the former does not discriminate against aliphatic alcohols on the basis of chain length, whereas the latter exhibits a clear bias on these grounds. In short, these results are encouraging with respect to inhibitor design, because both enzymes are able to accommodate a range of variants within their active site regions. Also encouraging are the clear differences in the active site specificities of these enzymes. This particular feature, when employed in combination with preferred sequence specificities, may ultimately provide a means to precisely target and inactivate specific protein kinases.

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