Molecular Rulers: An Assessment of Distance and Spatial Relationships of Src Tyrosine Kinase SH2 and Active Site Regions*

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The three-dimensional structures of the inactive conformations of Hck and Src, members of the Src protein-tyrosine kinase family, have recently been described. In both cases, the catalytic domain lies on the opposite face of the enzyme from the SH2 and SH3 domains. The active conformation of these enzymes has not yet been described. Given the known role of the SH2 and SH3 domains in promoting substrate binding, enzyme activation likely reorients the relative spatial arrangement between the SH2/SH3 domains and the active site region. We describe herein a series of “molecular rulers” and their use in assessing the topological and spatial relationships of the SH2 and active site regions of the Src protein-tyrosine kinase. These synthetic compounds contain sequences that are active site-directed (-Glu-Glu-Ile-Ile-(F₅)Phe-), where (F₅)Phe is pentafluorophenylalanine) and SH2-directed (-Tyr(P)-Glu-Glu-Ile-Glu-), separated by a sequence of variable length. The most potent bivalent compound, acetyl-Glu-Glu-Leu-Leu-(F₅)Phe-(GABA), displays a >120-fold enhancement in inhibitory potency relative to the simple monovalent active site-directed species, acetyl-Glu-Glu-Leu-Leu-(F₅)Phe-GABA (where GABA is γ-aminobutyric acid), displays a >120-fold enhancement in inhibitory potency relative to the simple monovalent active site-directed species, acetyl-Glu-Glu-Leu-Leu-(F₅)Phe-amide. The short linker length (3 GABA residues) between the active site- and SH2-directed peptide fragments suggests that the corresponding domains on the Src kinase can assume a nearly contiguous spatial arrangement in the active form of the enzyme.

Members of the Src kinase family are composed of a myristoylated N terminus, followed sequentially by a unique region, the SH3 domain, the SH2 domain, the catalytic domain, and the C-terminal tail containing a key regulatory site (Tyr₅₂⁷) by the SH2/SH3 domains are positioned, relative to the active site region, in a manner that promotes substrate binding in a catalytically competent fashion. What is the relative spatial orientation as well. The results described herein suggest that phosphorylated Tyr₅₂⁷ residue on the C-terminal tail, and the SH3 domain is associated with a proline-rich linker region between the SH2 and catalytic domains (2–6). These intramolecular binding events disrupt the formation of a critical salt bridge that is required for the coordination of ATP in the active site region. By contrast, in the active conformational state, the SH2 and SH3 domains actively promote protein-tyrosine kinase (PTK) activity via the formation of specific intermolecular protein-protein interactions with appropriate endogenous protein substrates, thereby docking these substrates to the PTK surface. In addition, the crystallographic data have led to subsequent studies that focused on key residues positioned in the sequence that links the catalytic and SH2 domains. For example, conversion of Trp₂⁶⁰ to Ala in both Hck and Src results in an increase in basal kinase activity (7). The hydrophobic Trp₂⁶⁰ residue not only interacts with the cChelix of the catalytic domain, but promotes the formation of the repressive intramolecular SH2 and SH3 contacts. In an analogous vein, Leu²⁵⁷ restricts the helix mobility, thereby blocking disassembly of the inactive Src kinase conformation (8). We do note that the structure of the active form of one Src kinase family member, namely Lck, has been determined (9). However, the structure solved in this case was that of the Lck catalytic domain, which lacks the SH2 and SH3 regions.

One of the most striking features of the Hck and Src kinase crystal structures is the orientation of the SH2 and SH3 domains relative to the active site region. The latter lies on the face of the enzyme molecule that is globally opposite to that of its regulatory domain counterparts. Given the clear role played by the SH2 and SH3 domains in substrate recognition, it is highly likely that the Src kinase family members undergo a major structural reorientation upon release from the catalytically repressed state. Presumably, in the active conformation, the SH2/SH3 domains are positioned, relative to the active site region, in a manner that promotes substrate binding in a catalytically competent fashion. What is the relative spatial orientation of the active site and SH2 regions in the active form of these enzymes? What is the preferred relationship (i.e. perpendicular, parallel, antiparallel, or some variation thereof) between amino acids sequences that are simultaneously bound to the active site and SH2 domains of these enzymes? We have addressed these questions by constructing bivalent ligands that can simultaneously associate with multiple regions on the Src kinase. These peptide-based species possess an SH2 recognition sequence linked, through a variable length tether of γ-aminobutyric acid (GABA) residues, to an active site-directed inhibitory peptide (10). Furthermore, we have prepared bivalent ligands that not only vary in tether chain length, but in spatial orientation as well. The results described herein sug-

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1 The abbreviations used are: PTK, protein-tyrosine kinase; GABA, γ-aminobutyric acid; Fmoc, N-(9-fluorenylmethoxycarbonyl; OtBu, tert-butoxy; (F₅)Phe, pentafluorophenylalanine; Ac, acetyl.
gest that the SH2 and active site regions of the Src kinase may very well be contiguous in the active form of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rink resin, Fmoc-protected amino acid derivatives, and all reagents for solid-phase peptide synthesis were obtained from Advanced Chem Tech. Fmoc-Tyr(Po benzoxyl)OH—OH was purchased from Novabiochem. 2-Methoxy-4-alkoxybenzyl alcohol and 4-(9-Fmoc-aminoanthen-3-yl)oxybutyryl]-4-methoxybenzhydrolyamide resins were obtained from Bachem. All other chemicals were purchased from Aldrich, except [125I]ATP (PerkinElmer Life Sciences), bovine serum albumin (Sigma), and ScintiSafe (Fisher). Phosphocellulose P-81 filter paper was acquired from Whatman, and SAM3000 biotin capture membrane was obtained from Promega. Enzyme assay solutions were prepared with deionized/distilled H2O.

**Human Receptor Signal—** Human Src was purchased from Upstate Biotechnology, Inc. The enzyme was expressed by recombinant baculovirus containing the human SRC gene in Sf9 insect cells and purified by the method of Bjorge et al. (11). The enzyme produced in this manner is not phosphorylated on its regulatory C-terminal tail (Tyre277) and consequently exists in an activated state.

**Preparation of H N-Glu(OrBu)-Glu(OrBu)-Leu-Leu-(Fmoc-
Tyr(PO3H2)) Glu(OrBu)-Glu(OrBu)-Leu-Leu-(Fmoc-
Tyr(PO3H2))Phe—** The protected active site-directed peptide Fmoc-Tyr(PO3H2)-Glu(OtBu)-Glu(OtBu)-Leu-Leu-(Fmoc-Tyr(PO3H2))-Phe was synthesized on Rink resin (substitution level = 0.34 mmol/g) utilizing a standard Fmoc solid-phase synthesis protocol on an Advanced Chem Tech 90 peptide synthesizer. The N terminus was deprotected, and tether construction and elongation were accomplished through multiple couplings of Fmoc-γ-aminoxylic acid. Aliquots of peptide resin were removed at the appropriate time in the synthetic sequence to provide tethers containing the desired number of γ-aminoxylic acid residues. The N-terminal Fmoc group was removed from each aliquot just prior to segment coupling.

**Preparation of H N-C-(CH2)3-COH-N-Tyr(Po-
H)-Glu(OrBu)-Glu(OrBu)-Ile-Glu(OrBu)-amide—** The protected peptide Fmoc-Tyr(Po-H)-Glu(OrBu)-Glu(OrBu)-Ile-Glu(OrBu) was synthesized on (4-Fmoc-aminoanthen-3-yl)oxybutyryl]-4-methoxybenzhydrolyamide resin (substitution level = 0.40 mmol/g). The N terminus was deprotected and acylated using 5 eq each of glutaric anhydride and N-methylmorpholine in methylene chloride. The protected peptide was cleaved from the resin using 1% trifluoroacetic acid in methylene chloride. Typically, 0.5–1 g of peptide-resin was incubated with 15 ml of 1% trifluoroacetic acid in methylene chloride for 15 min. The resin was isolated by filtration, and the filtrate was cooled to 0 °C and neutralized by the addition of N-methylmorpholine. The process was repeated several times to ensure complete removal of the peptide from the resin. The filtrate was evaporated to dryness, and the residue was dissolved in a small amount of methanol. The peptide fragment was precipitated with ethyl ether and isolated by filtration.

**Preparation of Type III Bivalent Inhibitors (Peptides 11–14)—** We employed a semisynthesis approach for the construction of the Type III inhibitors (Fig. 2). Typically, 2 eq of H O-C-(CH2)3-COHN-
Tyr(Po-H)-Glu(OrBu)-Glu(OrBu)-Ile-Glu(OrBu)-amide was condensed with 300 mg of the amino-terminal deprotected resin-bound peptide H N-(GABA)-Glu(OrBu)-Glu(OrBu)-Leu-Leu-(Phe) in the presence of 4 eq of benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate and N-hydroxybenzotriazole and 16 eq of N-methylmorpholine utilizing 2–3 ml of methylene chloride/N,N-dimethylformamide (1:1) as solvent. Couplings were allowed to proceed overnight at room temperature. The completeness of the segment condensations was monitored by the Kaiser test. Cleavage of the peptides from the resin with concomitant removal of all side chain-protecting groups was accomplished with 95% trifluoroacetic acid/H2O. The mixture was filtered, and the trifluoroacetic acid/H2O was removed under reduced pressure. The residue was dissolved in H2O, and the pH was adjusted to between 7 and 8. Crude peptides were purified by preparative reverse-phase high pressure liquid chromatography on a C18 column utilizing a linear gradient of CH3CN/H2O containing 0.1% trifluoroacetic acid. The appropriate fractions were combined and lyophilized.

**Preparation of Type II Bivalent Inhibitors (Peptides 7–10)—** Type II inhibitors were prepared by reaction of Rink resin with 2.0 eq of the solid-phase peptide synthesis protocol (10). In this case, Tyr(P) was incorporated into the peptides as the Fmoc-Tyr(Po benzoxyl)OH—OH derivative. Peptides were cleaved from the resin and purified as described above.

**Preparation of Bovine α1-Anti-trypsin—** The substrate was synthesized as previously described (10).

**Kinase Assays—** Assays were performed in triplicate at pH 7.5 in a thermostatted water bath maintained at 30 °C. For the determination of IC50 values, the following protocol was employed. Phosphorylation reactions were initiated by the addition of 10 μl of Src kinase from a concentrated stock solution to produce a final 40-μl solution containing [γ32P]ATP (final concentration varied and reported IC50 values: 100 μM [125I]ATP (500–1000 cpm/μmol), 30 μM Arg-Arg-Arg-
Arg-Arg-Ala-Glu-Glu-Glu-Tyr-NH-CH2CH2H2 substrate, 50 mM Tris, 10 mM MgCl2, 0.2 mg/ml bovine serum albumin, and 1.10 nM Src kinase. Reactions were terminated after 20 min by spotting 25-μl aliquots onto 2.1-cm diameter phosphocellulose paper discs. After 10 s, the discs were immersed in 10% glacial acetic acid and allowed to soak with swirling for 1 h. The acetic acid was decanted, and the discs were collectively washed with 4 volumes of 0.5% H3PO4 and 1 volume of water, followed by a final acetone rinse. The discs were air-dried, placed in plastic scintillation vials containing 3 ml of ScintiSafe, and subjected to scintillation counting for radioactivity.

**RESULTS AND DISCUSSION**

The crystal structures of two members of the Src tyrosine kinase family, namely Hck and Src, have been solved (12, 13). However, in both cases, it is the inactive conformation that has been elucidated. In the catalytically repressed state, the SH2 and SH3 domains lie globally opposite the ATP- and substrate-binding sites. However, once the enzyme is activated, the SH2 and SH3 domains serve as key participants in the recognition and subsequent phosphorylation of intracellular substrates. Indeed, the importance of the SH2 and SH3 domains in substrate recognition has been known for some time. For example, amino acid substitutions in the SH2 and/or SH3 domains of Src family kinases can modulate cellular transforming activity (14–16). In addition, although highly effective peptide-based active site-directed substrates and inhibitors have been repeatedly identified for Ser/Thr-specific protein kinases (17), analogous active site-directed species have, in general, proved extremely disappointing for the Tyr-specific protein kinases (18). One interpretation of these results is that PTKs, such as those of the Src family, recognize their substrates via SH2 (and other non-active site-associated) domains, a feature that simple active site-directed peptide substrates fail to recapitulate. These facts suggest that the SH2 domain may play a key role in positioning the protein substrate near the active site region of the Tyr kinase. Under these circumstances, it is reasonable to assume that the spatial relationship between the SH2 domain and the active site region undergoes a dramatic structural reorientation upon generation of the active enzyme form. In this study, we have examined the topological and spatial relationships between the SH2 and active site regions of the active form of the Src kinase using bivalent inhibitors, species that simultaneously recognize two separate binding sites on PTKs.

The strategy outlined in Fig. 1 employs a linear tether, composed of GABA amino acid residues, that serves as a link between SH2- and active site-targeted peptides. Both the active site and the SH2 domain recognize and bind to specific amino acid sequences contained within known Src kinase substrates. However, the highly simplistic illustration in Fig. 1 does not take into account the relative spatial orientation of the active site and the SH2 domain in the active form of the Src kinase, which is not presently known (12). For example, the spatial relationship of the active site and the SH2 domain may be such that the N terminus of the active site-directed peptide lies proximal to the C terminus of the SH2-targeted peptide (or vice versa). Other scenarios are possible as well. We have designed four structural variants of the bivalent ligand motif to assess these possibilities (Fig. 1). The bivalent ligand that is most structurally complementary to the preferred SH2 domain and active site spatial relationship in the active form of the Src kinase should serve as the most potent inhibitory species.

In the bivalent ligand of the general form I, the C terminus...
The bivalent inhibitors prepared in this study possess SH2- and active site-directed fragments that can simultaneously associate with the SH2 and active site regions of the Src kinase. Type I–IV bivalent ligands are defined by the relative orientations of the SH2- and active site-directed peptide fragments that can simultaneously associate with their targeted regions in a structurally compatible fashion. To address this possibility, we prepared the Type II and III analogs of Type I. We chose to limit the tether length in these bivalent ligand families to a maximum of 8 GABA units (or 7 GABA and 1 glutaric acid) units. The rationale for this decision was based upon the fact that those Type I inhibitors that possess tether lengths of 10 or more GABA subunits display a significant loss in inhibitory activity (Table I). This suggests that SH2- and active site-targeted peptide fragments that are separated by relatively long linker lengths act independently of each other.

The results obtained with the Type II and III inhibitors are provided in Table I (and graphically illustrated in Fig. 3). The monovalent peptide 1 exhibits an IC50 value of 1.6 mM, which is consistent with the poor inhibitory efficacy generally observed with simple active site-directed peptides targeted against Tyr-specific protein kinases. The best of the Type I inhibitors displays an 85-fold enhanced inhibitory potency for the Src kinase relative to peptide 1. Furthermore, there is a clear correlation between inhibitory efficacy and GABA chain length. By contrast, the most potent Type III inhibitor is little more than 8-fold better than the active site-directed control peptide 1. In

Fig. 1. General structures of Src kinase-targeted bivalent ligands. The bivalent inhibitors prepared in this study possess SH2- and active site-directed fragments that can simultaneously associate with the SH2 and active site regions of the Src kinase. Type I–IV bivalent ligands are defined by the relative orientations of the SH2- and active site-targeted fragments.
Inhibitory constants were determined as described under "Experimental Procedures." The values are given as the means ± S.D. Ga, glutaric acid.

| Peptide | Bivalent inhibitor | IC₅₀ (µM) |
|---------|--------------------|----------|
| 1       | Ac-Glu-Glu-Leu-(F₅)Phe-amide | 1590 ± 170 |
| Type I (2) | Ac-Tyr(P)-Glu-Glu-Ile-Glu-(GABA)₆-Glu-Glu-Leu-(F₅)Phe-amide | 195 ± 26 |
| Type I (3) | Ac-Tyr(P)-Glu-Glu-Ile-Glu-(GABA)₆-Glu-Glu-Leu-(F₅)Phe-amide | 35 ± 5 |
| Type I (4) | Ac-pTyr-Glu-Glu-Ile-Glu-(GABA)₆-Glu-Glu-Leu-(F₅)Phe-amide | 18.5 ± 1 |
| Type I (5) | Ac-Tyr(P)-Glu-Glu-Ile-Glu-(GABA)₆-Glu-Glu-Leu-(F₅)Phe-amide | 250 ± 25 |
| Type I (6) | Ac-Tyr(P)-Glu-Glu-Ile-Glu-(GABA)₆-Glu-Glu-Leu-(F₅)Phe-amide | 400 ± 50 |
| Type II (7) | Ac-Glu-Glu-Leu-(F₅)Phe-(GABA)₆-Tyr(P)-Glu-Glu-Ile-Glu-amide | 175 ± 20 |
| Type II (8) | Ac-Glu-Glu-Leu-(F₅)Phe-(GABA)₆-Tyr(P)-Glu-Glu-Ile-Glu-amide | 35 ± 2 |
| Type II (9) | Ac-Glu-Glu-Leu-(F₅)Phe-(GABA)₆-Tyr(P)-Glu-Glu-Ile-Glu-amide | 100 ± 15 |
| Type II (10) | Ac-Glu-Glu-Leu-(F₅)Phe-(GABA)₆-Tyr(P)-Glu-Glu-Ile-Glu-amide | 90 ± 20 |
| Type III (11) | amide-ulg-ulg-ulG-(PyT-Ga-GABA-Glu-Glu-Leu-(F₅)Phe-amide | 200 ± 50 |
| Type III (12) | amide-ulG-ulg-ulG-(PyT-Ga-GABA-Glu-Glu-Leu-(F₅)Phe-amide | 430 ± 20 |
| Type III (13) | amide-ulG-ulg-ulG-(PyT-Ga-GABA-Glu-Glu-Leu-(F₅)Phe-amide | 230 ± 30 |
| Type III (14) | amide-ulG-ulg-ulG-(PyT-Ga-GABA-Glu-Glu-Leu-(F₅)Phe-amide | 180 ± 60 |
| Type III (15) | Ac-Glu-Glu-Leu-(F₅)Phe-(GABA)₆-Tyr(P)-Glu-Glu-Ile-Glu-amide | 13 ± 1 |
| Type III (16) | Ac-Glu-Glu-Leu-(F₅)Phe-(GABA)₆-Tyr(P)-Glu-Glu-Ile-Glu-amide | 28 ± 2 |

* The Tyr(P)-containing SH2-directed component of the Type III ligands is written backwards to emphasize the antiparallel nature of these ligands.

Fig. 2. Synthesis of the Type III bivalent Src kinase inhibitors. See "Results and Discussion" for additional details. pTyr and pTyrT; Tyr(P); Bu, t-butyl; PyBOP, benzotriazol-1-yloxytritylpyrrolidinophosphonium hexafluorophosphate; HOBt, N-hydroxybenzotriazole; NMM, N-methylmorpholine.

Fig. 3. Inhibitory potency (IC₅₀) of Type I (●), II (△), and III (■) inhibitors versus tether length (number of GABA or GABA + glutaric acid residues).

addition, there is a clear absence of any relationship between inhibitory potency and GABA chain length. In an optimized bivalent ligand, the high affinity SH2-directed fragment should deliver the peptides to the Src kinase at or better than low micromolar concentrations. The results imply that the overall structural arrangement of the SH2- and active site-targeted fragments in Type III inhibitors is not complementary to the relative spatial orientation of the SH2 and active site modules on the enzyme surface. Under these circumstances, kinase inhibition likely occurs only if the bivalent peptides assume a less than energetically favorable enzyme-bound conformation, which would account for the modest inhibitory profile of the Type III class of inhibitors. By contrast, the results obtained for the Type II inhibitors reveal an obvious trend. Inhibitory potency improves as a function of increasing tether length up to 4 GABA residues (36 µM). Peptide 8 is a 46-fold more potent inhibitor of Src kinase than peptide 1. Inhibitory potency appears to plateau at a tether length of 6 GABA units, with peptides 9 and 10 being equipotent.

Previous studies have demonstrated that simple peptides with a special affinity for either the SH2 or SH3 domain promote the catalytic activity of Src kinase family members. For example, Miller and co-workers (22) have shown that the SH2 ligand, Tyr(P)-Glu-Ile, enhances the catalytic activity of Hck by 2.5-fold as assessed by the phosphorylation of the simple monovalent active site-directed peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-His-Tyr-Ala-Ala-Arg-Gly. The physical interpretation of this observation is that the Tyr(P)-bearing peptide partially releases the enzyme from its catalytically repressed state, a state in which the SH2 domain is intramolecularly associated with phosphorylated Tyr527. Furthermore, Miller and co-workers have demonstrated that peptides bearing the -Tyr(P)-Glu-Ile- motif specifically associate only with the SH2-binding region of the Src kinase, an observation that is true with v-Src as well, the constitutively active form of the Src kinase (23). Based upon these previously reported observations, the enhanced affinity displayed by the bivalent inhibitors described in this report is likely due to the simultaneous occupancy of both SH2 and active site regions. If SH2 domain occupancy is the key component of bivalent ligand activity, then any species that specifically occludes the SH2 domain should also reduce the inhibitory potency of these bivalent ligands. As noted above, the -Tyr(P)-Glu-Ile- sequence associates only with the SH2 domain (and no other region and/or site) of the Src kinase. Indeed, in the presence of 300 µM...
Ac-Tyr(P)-Glu-Glu-Ile, the IC\textsubscript{50} of peptide 8 is markedly improved (180 ± 10 \textmu M \textit{versus} 35 ± 2 \textmu M in the absence of the Tyr(P) peptide).

Can inhibitors more potent than peptide 8 be constructed by fine-tuning the tether length even further? We prepared peptides 15 and 16, species that contain 3 and 5 GABA units, respectively. As is evident from Table I, peptide 15 is the best of the bivalent Src kinase inhibitors prepared to date, displaying an IC\textsubscript{50} value nearly 120-fold better than peptide 1. Clearly, an array of additional refinements are possible, such as replacing a single GABA subunit in the tether contained in peptide 15 with 5-aminovaleric acid (one carbon more than GABA), \beta-alanine (one carbon less than GABA), or glycine (two carbons less than GABA), which will alter the overall tether length by a few angstroms. However, as is evident from the results displayed in Table I, the potency of the bivalent inhibitors appear to be asymptotically approaching the known affinity of the -Tyr(P)-Glu-Glu-Ile- SH2-targeting sequence contained within all of these inhibitors.

The identification of Type II peptide 15 as an effective inhibitor of Src kinase provides insight into the spatial relationship between the SH2 and active site regions of the active enzyme form. The optimal tether length for Type I inhibitors is 8 GABA residues. Furthermore, the overall distance between the Tyr(P) moiety in the SH2-directed segment and (F\textsubscript{5})Phe in the active site-directed segment is considerable. If the 8 GABA residue-containing peptide 4 assumes an extended conformation when bound to the enzyme surface, one could draw the conclusion that the Tyr(P) and (F\textsubscript{5})Phe recognition sites are separated by >40 Å. However, by reorienting the SH2 and active site-directed sequences, we have obtained a peptide (peptide 15) in which the tether length has been more than halved. Perhaps even more noteworthy is the dramatic reduction in the distance between the critical Tyr(P) moiety of the SH2 recognition sequence and the key pentafluorophenylalanine Tyr surrogate of the active site-directed sequence. This is abundantly clear by comparing the best Type II inhibitor, peptide 15 (IC\textsubscript{50} = 13.4 \mu M), with its Type I counterpart, peptide 4 (IC\textsubscript{50} = 18.5 \mu M). The distance between Tyr(P) and (F\textsubscript{5})Phe in peptide 4 is 16 amino acid residues. By contrast, this distance is only 3 residues in peptide 15. Indeed, even if peptide 15 assumes an extended conformation when enzyme-bound, the distance between the key Tyr(P) and (F\textsubscript{5})Phe recognition sites is ~10–12 Å. This strongly implies that the catalytic and SH2 regions of the Src kinase undergo a considerable structural reorganization as the enzyme assumes the catalytically active state. Furthermore, the modest linear distance between the key Tyr(P) and (F\textsubscript{5})Phe moieties in peptide 15 suggests that the SH2 and active site regions can assume a nearly contiguous spatial arrangement in the catalytically active form of the enzyme, implying that the -Tyr(P)-Glu-Glu-Ile-binding portion of the SH2 domain could very well serve as an extension of the active site region itself.

In addition to the inhibitors described in this study, Type IV ligands could have been examined. Like their Type III counterparts, Type IV compounds contain SH2 and active site recognition elements that are oriented antiparallel to each other (Fig. 1). However, it is the C termini of these recognition elements, rather than the N termini, that are appended to the GABA-based tether. This relative orientation assures that the critical Tyr(P)-to-(F\textsubscript{5})Phe distance will always be greater for the Type IV analogs than that obtained with peptide 15. Consequently, we chose not to explore the preparation of this class of bivalent inhibitor.

Finally, we note that the bivalent approach outlined herein represents an alternative strategy for the acquisition of potentially selective and potent Tyr kinase inhibitors. The majority of the peptide-based active site-directed inhibitors described to date have been prepared by replacing the phosphorylatable tyrosine residue with a phenylalanine moiety. This approach has, in general, led to the production of relatively poor inhibitory species. A portion of the poor inhibitory activity of these compounds can be attributed to the missing phenolic hydroxyl, which likely facilitates productive active site interactions. Indeed, the most potent peptide-based active site-directed PTK inhibitors are those in which the phosphorylatable tyrosine has been replaced by a non-phosphorylatable phenolic analog such as l-Dopa (24) or tetrafluorotyrosine (25). In addition, the low affinity displayed by simple active site-directed peptides may be due to the fact that they fail to reproduce the mechanism by which intact endogenous protein substrates are recognized by PTKs (i.e. via non-active site regions such as SH2 domains (1, 26, 27)). In short, the Tyr(P)-SH2 domain interactions occur with a significantly higher affinity than those that transpire in the active site region. Based on this notion, Miller and co-workers (28) have investigated the Hck-catalyzed phosphorylation of peptide substrates possessing an SH2 recognition sequence. The most potent of these substrates display as much as a 10-fold reduction in their \(K_m\) value relative to peptides that lack the Tyr(P)-bearing SH2 recognition element, which presumably reflects the enhanced binding of the peptides to the Hck surface (28). Cowburn and co-workers (29) have constructed “consolidated ligands” that simultaneously bind to the SH2 and SH3 domains of the Abelson PTK with enhanced affinity. Recently, these investigators also prepared subfamilies of these ligands that contain different relative orientations of the SH2- and SH3-directed sequences (30). The general structure of their tightest binding ligand is (SH2-directed ligand)-tether-(SH3-directed ligand), where the tether is composed of 7 Gly residues. Since members of the Src kinase family also contain SH3 domains, it may be possible to ultimately prepare trivalent ligands that are able to simultaneously associate with the three key binding domains of PTKs. Finally, we note that Pluskey and co-workers (31) tethered two phosphotyrosyl peptides together via aminohexanoic acid linkages to produce bivalent ligands that bind to the two SH2 domains of SH-PTP2. The latter were found to stimulate catalytic activity in a more pronounced fashion than simple monovalent SH2-targeted phosphopeptides. As noted above, simple monovalent ligands that associate with the SH2 or SH3 regulatory sites of Src kinase family members often promote enhanced enzymatic activity. By contrast, the bivalent inhibitors described in this study behave in a decidedly different fashion. These species are both bivalent and bifunctional. Not only do these peptides coordinate to the SH2 domain of Src, which should block the assembly of Src-based signaling complexes, but they shut down the catalytic activity of the enzyme as well.

In summary, we have employed bivalent ligands to investigate the distance and spatial relationships between the SH2 and active site regions of the Src PTK. The most potent bivalent species identified in this study is peptide 15, which indicates that there is a high degree of structural complementarity between the sequence arrangement in peptide 15 and the preferred active conformation of the Src kinase itself. The close linear proximity of the key Tyr(P) and (F\textsubscript{5})Phe residues in peptide 15 implies that the SH2 domain may very well serve as an extension of the active site when the Src kinase assumes the active, catalytically competent, conformational state.

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