Identification of residues involved in v-Src substrate recognition by site-directed mutagenesis

Noriko Yokoyama, W. Todd Miller*

Department of Physiology and Biophysics, School of Medicine, State University of New York, Stony Brook, NY 11794-8661, USA

Received 6 July 1999

Abstract To study the role of the catalytic domain in v-Src substrate specificity, we engineered three site-directed mutants (Leu-472 to Tyr or Trp and Thr-429 to Met). The mutant forms of Src were expressed in Sf9 cells and purified. We analyzed the substrate specificities of wild-type v-Src and the mutants using two series of peptides that varied at residues C-terminal to tyrosine. The peptides contained either the YMTM motif found in insulin receptor substrate-1 (IRS-1) or the YGEF motif identified from peptide library experiments to be the optimal sequence for Src. Mutations at positions Leu-472 or Thr-429 caused changes in substrate specificity at positions P+1 and P+3 (i.e. one or three residues C-terminal to tyrosine). This was particularly evident in the case of the L-472W mutant, which had pronounced alterations in its preferences at the P+1 position. The results suggest that residue Leu-472 plays a role in P+1 substrate recognition by Src. We discuss the results in the light of recent work on the roles of the SH2, SH3 and catalytic domains of Src in substrate specificity.

Key words: Tyrosine kinase; Mutagenesis; v-Src; Peptide substrate specificity

1. Introduction

c-Src is a cytoplasmic tyrosine kinase that is widely expressed in mammalian tissues. c-Src participates in signal transduction pathways that transmit extracellular signals across the cell membrane to the cytoplasm and nucleus (reviewed in [1]). v-Src, the viral homolog of c-Src, is the protein product of the transforming gene from Rous sarcoma virus. Src kinases are composed of an N-terminal membrane binding region, a unique domain, an SH3 domain that binds proline-rich sequences, a phosphotyrosine binding SH2 domain, a tyrosine kinase catalytic domain and a C-terminal tail, which is closely involved in the negative regulation of kinase activity [2,3].

The recent crystal structures of the inactive forms of Src and Hck, a Src-family kinase, reveal that intramolecular interactions involving the SH3 and SH2 domains inhibit kinase activity [4,5]. A polyproline type II helix is present in the linker connecting the SH2 domain to the catalytic domain. This linker acts as a docking site for the SH3 domain. This SH3-PPII linker interaction appears to inhibit kinase activity by stabilizing an inactive conformation of the catalytic domain [4–6]. Binding of the SH2 domain to the phosphorylated C-terminal tail also contributes to enzyme inhibition [1–6].

In addition to their inhibitory roles, the SH2 and SH3 domains of Src-family kinases have been shown to mediate binding of Src to other proteins, including some of its substrates. For example, Src and Abl tyrosine kinases preferentially phosphorylate peptides that are suited to bind their own SH2 domains [7]. The presence of a high-affinity SH2 binding site in a peptide substrate results in a dramatic increase in the phosphorylation rate of additional tyrosines in the peptide [8]. Replacement of the Abl SH2 domain with other SH2 domains led to a change in substrate specificity in vivo [9]. These results suggest that the SH2 domains of intracellular kinases may be involved in substrate recognition.

At present, the role of the catalytic domains of tyrosine kinases in determining target specificity is not fully understood. The crystal structure of the insulin receptor complexed with peptide substrate highlights specific interactions with residues surrounding tyrosine in the substrate [10]. Such interactions could contribute to substrate specificity for other tyrosine kinases as well. This is illustrated by studies of the RET receptor protein kinase. Wild-type RET receptor tyrosine kinase has a methionine residue at position 918, whereas cytoplasmic tyrosine kinases have threonine at the corresponding position. A naturally occurring Met-918→Thr mutation in the RET receptor is associated with multiple endocrine neoplasia type 2B [11,12]. This variant of the kinase preferentially phosphorylates optimal substrates for Src and Abl, whereas wild-type RET protein preferentially phosphorylates the optimal peptide substrates for EGF receptor [7]. This result highlights the importance of the catalytic domain in tyrosine kinase signaling. Taken together with studies of the SH2 domains described above, it appears that the catalytic and SH2 domains provide a double selection to maintain the fidelity of signaling events. In this paper, we describe experiments aimed at understanding the contribution of the Src catalytic domain itself in substrate specificity.

Previously, we generated site-directed mutants of the Abl catalytic domain that showed distinct changes in substrate specificity relative to wild-type Abl [13]. In this paper, we have applied a similar protein engineering approach to v-Src kinase to study the enzyme's substrate specificity. Based on the results with site-directed mutants of Abl, we have engineered three mutant forms of v-Src kinase to investigate changes in substrate specificity. We assayed the effects of mutagenesis using synthetic peptide substrates. Synthetic peptides derived from the insulin receptor kinase substrate-1 (IRS-1) containing Tyr-Met-X-Met (YMXM) motifs are good substrates for v-Src and v-Abl[14]. Moreover, these non-receptor tyrosine kinases show distinct specificities towards the YMXM motif peptides. Another excellent substrate for Src is a synthetic peptide possessing the sequence AEEEIYGEFEAKKKK (YGEF motif) that was identified in peptide li-

*Corresponding author. Fax: (1) (516) 444 3432.
E-mail: miller@physiology.pnb.sunysb.edu

0014-5793/99/$20.00
PII: S0014-5793(99)00992-8

brary experiments [7]. Therefore, we prepared two series of peptides containing variations in the YMXM and YGEF motifs to examine the substrate specificities of our engineered mutant forms of v-Src.

2. Materials and methods

2.1. v-Src expression plasmid

v-Src DNA from the Schmidt-Ruppin strain of Rous sarcoma virus (encoding amino acids 77–526, comprising the SH3, SH2 and catalytic domains of v-Src) was subcloned from a pGe×2T-SH3-SH2-catalytic domain plasmid [15] into pFastBacHTb (Gibco BRL) as a *Bam*HI/*Eco*RI fragment.

2.2. Mutagenesis, expression in Sf9 cells and purification of v-Src

We carried out site-directed mutagenesis using the Quikchange Mutagenesis System from Stratagene. For each mutation, the pFastBacHTb-Src plasmid and two synthetic oligonucleotides containing the desired mutation in v-Src kinase were used. The DNA sequences encoding the catalytic domains of mutant forms of v-Src were confirmed by DNA sequencing.

Wild-type and mutant forms of v-Src were expressed in *Spodoptera frugiperda* (Sf9) cells. 0.5 l of Sf9 cells (1.5×10^6 cells/ml) was infected with recombinant v-Src baculovirus and cells were harvested 3 days after infection for protein purification. Cells were washed with phosphate-buffered saline and lysed in a French pressure cell two times, in buffer containing 10 mM Tris-HCl (pH 8.5), 50 mM sodium fluoride, 0.2 mM sodium vanadate, 1% NP-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 2 mM phenylmethylsulfonyl fluoride. After centrifugation at $10\,000\times g$ for 30 min, the supernatant was applied to a hydroxyapatite column (2.5×3 cm) which was pre-equilibrated with homogenization buffer. Src kinases were eluted by a gradient of potassium phosphate (0–0.4 M) in homogenization buffer. Src activity was measured with KKEEEEIYMMMM (E_4IYM_4) as a peptide substrate and fractions containing Src activity were pooled and dialyzed against 20 mM Tris-HCl (pH 8.5), 500 mM KCl, 5 mM 2-mercaptoethanol, 20 mM imidazole and 10% glycerol. After dialysis, fractions were applied to a Ni-NTA column (bed volume 3 ml, Qiagen) which was pre-equilibrated with 20 mM Tris-HCl (pH 8.5), 100 mM KCl, 5 mM 2-mercaptoethanol, 20 mM imidazole and 10% glycerol. Src kinases were eluted by 20 mM Tris-HCl (pH 8.5), 100 mM KCl, 100 mM imidazole, 5 mM 2-mercaptoethanol and 10% glycerol.

2.3. Peptide synthesis and characterization

Peptide substrates were synthesized on an Applied Biosystems automated peptide synthesizer using standard Fmoc chemistry [16]. Peptides were purified by reversed-phase high performance liquid chromatography and characterized by matrix-assisted laser description/ionization time of flight mass spectrometry. Quantitative amino acid analysis (Commonwealth Biotechnologies) was used to determine the concentration of peptide stock solutions.

2.4. Kinase assays using synthetic peptide substrates

Kinase activity was measured in two ways: (1) using the phosphocellulose binding assay [17] or (2) a continuous spectrophotometric assay [18]. For initial comparisons of substrate specificity using the phosphocellulose binding assay, the reaction mixtures contained 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.25 mM ATP, 0.5 mM peptide substrate and [γ-^{32}P]ATP (100–200 cpm/pmol). Reactions were initiated by addition of Src. After a 30 min incubation at 30°C, reactions were stopped and spotted on phosphocellulose paper. Incorporation of ^{32}P into peptide was determined by liquid scintillation counting. A continuous spectrophotometric assay [18] was used for a more detailed kinetic analysis. Reactions were carried out at 30°C in volumes of 500 μl containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.25 mM ATP, 1 mM phosphoenolpyruvate, 1 mg α-nicotinamide adenine dinucleotide reduced form, pyruvate kinase (42 U), lactate dehydrogenase (60 U) and various concentrations of peptides (60–2000 μM). Reactions were initiated by the addition of Src or Src mutants. Data were collected every 30 s to determine initial rates of peptide phosphorylation.

3. Results and discussion

3.1. Src residues targeted for mutagenesis

In a previous report from this laboratory, mutation of seven residues in c-Abl caused changes in substrate specificity, especially in the recognition of the P−1, P+1 and P+3 positions of bound peptide substrates [13]. (Amino acids in protein kinase substrates are numbered relative to P, the phosphorylated residue). Src and Abl display differences in substrate specificity at the P+1 and P+3 positions. Based on the known three-dimensional structure of Src [4] and by analogy to our results for Abl [13], we selected Leu-472 as a residue that might be involved in P+3 substrate recognition. We constructed two mutations at this position: Leu-472 to Trp (L-472W), because a Trp residue at this position in Abl altered P+3 specificity [13], and Leu-472 to Tyr (L-472Y), the corresponding residue in wild-type Abl. We note that amino acids near the putative P+1/P+3 pocket in the Hck and Src structures are conserved [4,5], supporting the idea that local perturbations in structure will have a predictable effect on specificity. A naturally occurring mutation of the kinase domain of RET receptor (Met-918→Thr) showed alterations in P+1 position substrate recognition [7,11,12]. Therefore, we prepared a Src mutant in which the corresponding amino acid (Thr-429) was changed to Met, the residue found in receptor tyrosine kinases. We expected that this mutation might affect substrate recognition at the P+1 position. The positions in v-Src that were mutated are shown in Fig. 1.

3.2. Expression of wild and mutant forms of v-Src in Sf9 cells

The Src construct used in this study contained the SH3, SH2 and catalytic domains. Wild-type and mutant forms of Src from baculovirus-infected Sf9 cells were purified by a hy-

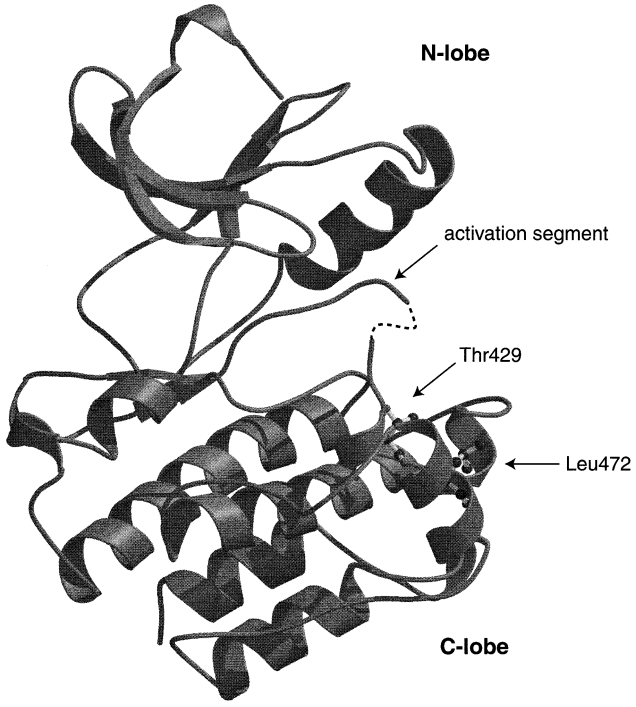

Fig. 1. Ribbon representation of the Src tyrosine kinase catalytic domain structure [4]. The residues that we mutated are shown in ball and stick format. The figure was generated from the atomic coordinates using the program MOLSCRIPT [19].

Table 1
Peptide sequences used in this study

1. Sequences derived from IRS-1 Tyr-987	
YMTM	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Met-Thr-Met-Gln-Ile-Gly
YNleTM	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Nle-Thr-Met-Gln-Ile-Gly
YETM	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Glu-Thr-Met-Gln-Ile-Gly
YITM	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Ile-Thr-Met-Gln-Ile-Gly
YATM	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Ala-Thr-Met-Gln-Ile-Gly
YMTA	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Met-Thr-Ala-Gln-Ile-Gly
YMTP	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Met-Thr-Pro-Gln-Ile-Gly
YMTT	Lys-Lys-Ser-Arg-Gly-Asp-**Tyr**-Met-Thr-Thr-Gln-Ile-Gly
2. Sequences derived from Src optimal phosphorylation motif	
YGEF	Ala-Glu-Glu-Glu-Ile-**Tyr**-Gly-Glu-Phe-Glu-Ala-KKKKG
YAEF	Ala-Glu-Glu-Glu-Ile-**Tyr**-Ala-Glu-Phe-Glu-Ala-KKKKG
YMEF	Ala-Glu-Glu-Glu-Ile-**Tyr**-Met-Glu-Phe-Glu-Ala-KKKKG
YGET	Ala-Glu-Glu-Glu-Ile-**Tyr**-Gly-Glu-Thr-Glu-Ala-KKKKG
YGEA	Ala-Glu-Glu-Glu-Ile-**Tyr**-Gly-Glu-Ala-Glu-Ala-KKKKG
YGEM	Ala-Glu-Glu-Glu-Ile-**Tyr**-Gly-Glu-Met-Glu-Ala-KKKKG
3. Other sequences	
E_4YM4	Lys-Lys-Glu-Glu-Glu-Glu-**Tyr**-Met-Met-Met-Met
E_4IYM_4	Lys-Lys-Glu-Glu-Glu-Glu-Ile-**Tyr**-Met-Met-Met-Met

The names used in the text are given in the left-hand column. The phosphorylated tyrosine residues are in bold. The underlined residues are changes from the parent sequences. KKKKG = Lys-Lys-Lys-Lys-Gly. Nle = norleucine.

droxyapatite column followed by a Ni-NTA column. (Initial attempts to use Ni-NTA as a single step purification yielded insufficiently pure preparations of enzymes). The proteins were more than 95% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. The proteins also displayed immunoreactivity towards anti-c-Src antibody (results not shown).

3.3. Comparison of wild-type and mutant forms of v-Src with IRS-1 (YMXM) and YGEF peptides

Previously, we demonstrated that peptides containing YMXM motifs derived from IRS-1 are good substrates for v-Src and c-Abl [14]. For initial comparative experiments, we compared the in vitro substrate specificities of the wild-type and mutant forms of v-Src using a series of IRS-1 synthetic peptides containing YMXM motifs (Table 1). These peptides were based on the sequence of IRS-1 surrounding Tyr-987 (SRGDYMTMQ) with single amino acid substitutions at the P+1/P+3 positions. For insulin receptor, these phosphorylated YMTM motifs act as docking sites for downstream signalling proteins containing SH2 domains [20]. As shown in Fig. 2, IRS-1 peptides are substrates for the mutant forms of Src, although the specific activities of the Src mutants towards these peptides were typically $\approx$10 times lower than that of wild-type v-Src. Nonetheless, some differences in substrate specificity were apparent. Wild-type v-Src showed the highest specific activity towards peptides containing substitutions at the P+1 position such as YATM, YETM and YNleTM, whereas peptides containing substitutions at the P+3 position such as YMTT and YMTP showed much lower specific activities. The L-472Y mutant preferred Nle or Ile at the P+1

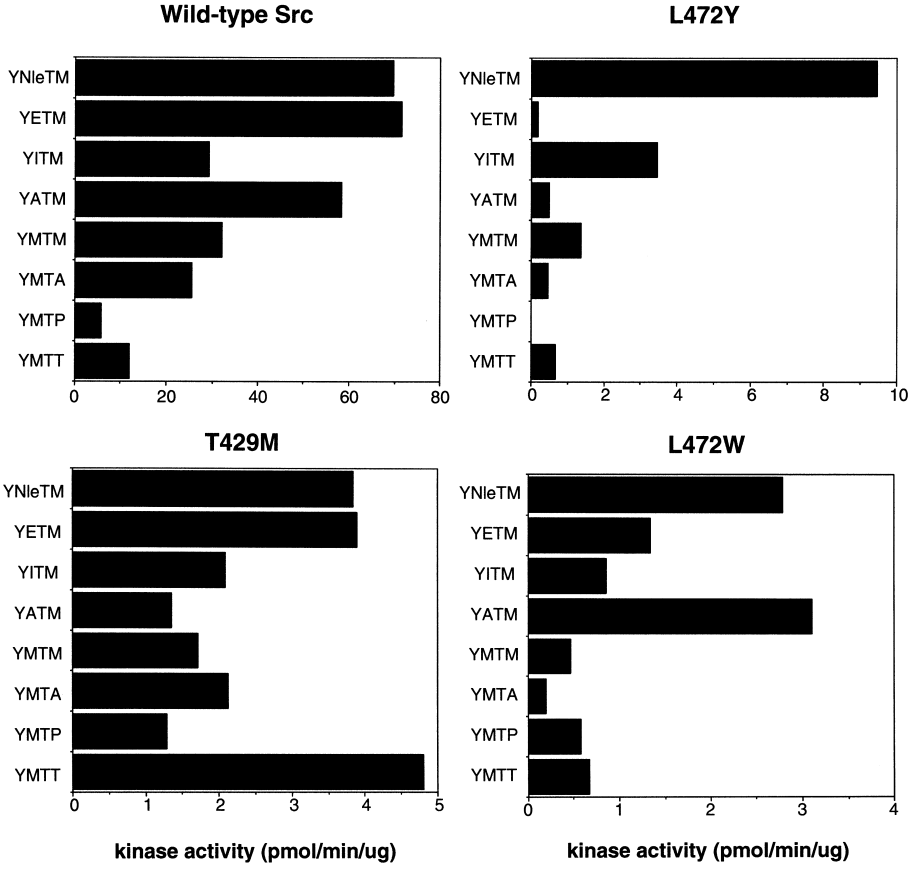

Fig. 2. Phosphorylation of IRS-1 peptides by wild-type and three mutant forms of v-Src (T-429M, L-472W and L-472Y). The Src kinase activities were determined after 30 min incubation at 30°C using the phosphocellulose binding assay [17]. The peptide concentration in the assay was 0.5 mM. The unit of specific activity is pmol of phosphate incorporated/min/μg protein. The sequences of the peptides are given in Table 1.

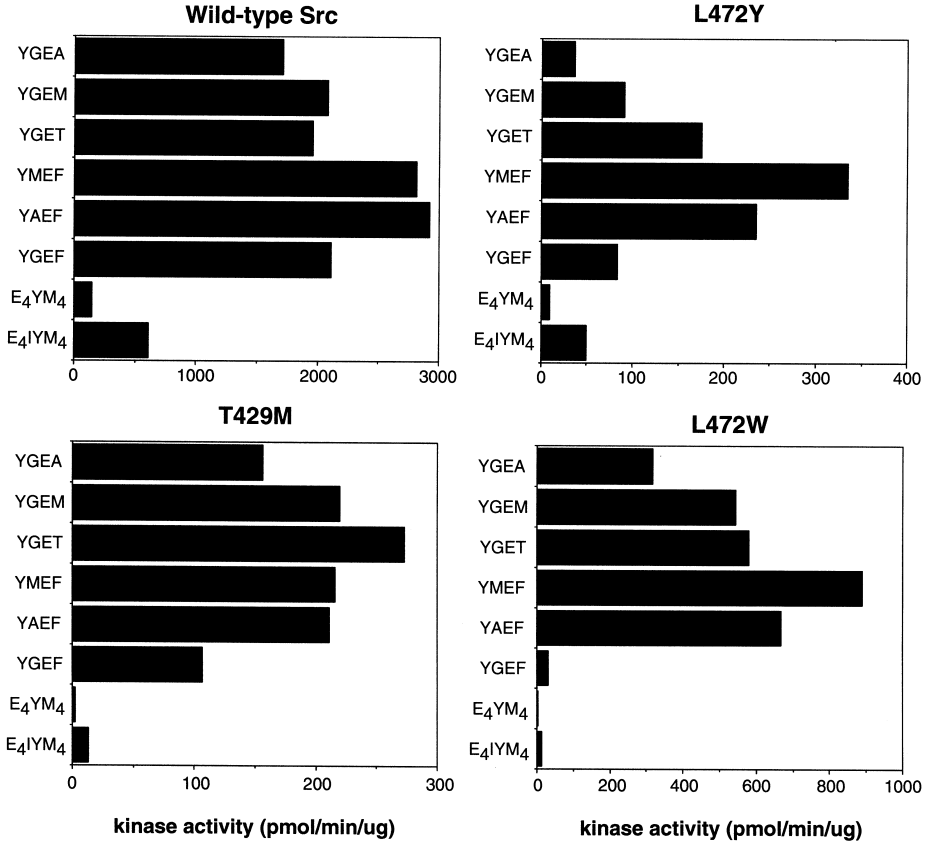

Fig. 3. Phosphorylation of YGEF peptides by wild-type and three mutant forms of v-Src (T-429M, L-472W and L-472Y). The activities of wild-type and mutant forms of Src were determined using a peptide concentration of 0.5 mM after 30 min incubation at 30°C. Data were obtained using the phosphocellulose binding assay [17]. The unit of specific activity is pmol of phosphate incorporated/min/μg protein. The sequences of the peptides are given in Table 1.

position, but a peptide containing Glu at the P+1 position was a poor substrate (Fig. 2). The L-472W form of Src preferred YATM and YNleTM substituted peptides. The T-429M mutant preferred a peptide containing Thr at the P+3 position (YMTT), although this sequence was not recognized well by wild-type Src or the other mutants. These data suggested that mutations at residues Leu-472 or Thr-429 of Src caused changes in recognition of the P+1/P+3 positions in bound peptide substrates. Src is more tolerant of substitutions at the P+1 position of YMXM-containing substrates than insulin receptor, another kinase that has been examined in detail with regard to phosphorylation of YMXM peptides [21]. This may explain why the changes we observed in substrate specificity were subtle and were reflected in changes in the rank order of specific activities (Fig. 2), rather than in complete changes in the overall specificity.

To examine changes in substrate specificity in a different peptide context, we synthesized another series of peptides based on the parent sequence, AEEEIYGEFEAKKKK. This peptide contains YGEF, the optimal Src phosphorylation sequence C-terminal to tyrosine that was identified in peptide library studies [7]. We designed peptides with substitutions at the P+1 and P+3 positions of YGEF based on our results from the IRS-1 peptides (Table 1). As shown in Fig. 3, in initial screens of activity, YGEF and its derivatives displayed much higher specific activities for all v-Src kinases than the IRS-1-derived peptides. For wild-type Src, the specific activity of the best YGEF peptide was ≈35 times higher than the best YMTM peptides (compare Figs. 2 and 3). Substitutions of Ala or Met at the P+1 position in the YGEF motif resulted in higher specific activities for wild-type and mutant forms of v-Src. This effect was especially pronounced for the L-472W mutant of Src, where substitution of Met or Ala at the P+1 position increased the specific activity 29- or 22-fold, respectively, relative to YGEF (Fig. 3). The other mutants (L-472Y and T-429M) also showed a more dramatic difference between the YMEF/YAEF peptides and the YGEF peptide than wild-type Src did (Fig. 3). KKEEEEYMMMM (E_4YM_4), the optimal peptide substrate predicted for insulin receptor [7], was a poorer substrate for all v-Src kinases (Fig. 3). Taken together, the data suggested that mutations of Thr-429 and particularly of Leu-472 cause alterations in substrate preference at the P+1 position.

3.4. Kinetic parameters of the phosphorylation of YGEF-containing peptide substrates by wild-type and mutant forms of v-Src

To further investigate the substrate specificity of wild-type Src and the mutants, kinetic analysis was carried out using YGEF peptides with substitutions at the P+1 or P+3 positions (YGEF, YAEF, YMEF and YGET) (Table 2). An additional peptide, KKEEEEIYMMMM (E_4IYM_4), was also tested. This peptide is unrelated in sequence to the YGEF substrates at positions N-terminal to tyrosine, but possesses

Table 2
Kinetic parameters for phosphorylation of peptide substrates by wild-type and mutant forms of Src

	K_m (μM)	V_{max} (μmol/min/mg)	$V_{max}/K_m \times 10^3$
Wild-type Src			
YGEF	98 ± 24	120.3 ± 9.5	1227
YAEF	108 ± 44	29.2 ± 2.8	270
YMEF	217 ± 49	4.1 ± 2.8	18.9
YGET	185 ± 60	26.4 ± 2.3	142.7
E_4IYM_4	247 ± 72	36.4 ± 3.0	147.3
T-429M			
YGEF	53 ± 22	1.8 ± 0.2	33.9
YAEF	470 ± 21	2.6 ± 0.4	5.5
YMEF	162 ± 66	0.8 ± 0.11	4.9
YGET	120 ± 46	1.1 ± 0.1	9.2
E_4IYM_4	153 ± 79	1.2 ± 0.09	7.8
L-472W			
YGEF	141 ± 44	1.7 ± 0.16	12.0
YAEF	100 ± 70	3.0 ± 0.58	30.0
YMEF	259 ± 159	6.1 ± 1.1	23.6
YGET	208 ± 92	0.6 ± 0.08	2.9
E_4IYM_4	180 ± 93	0.6 ± 0.07	3.3
L-472Y			
YGEF	65 ± 15	3.4 ± 0.06	52.3
YAEF	172 ± 60	1.4 ± 0.15	8.1
YMEF	236 ± 98	1.0 ± 0.13	4.2
YGET	255 ± 93	1.7 ± 0.22	6.7
E_4IYM_4	298 ± 32	0.7 ± 0.09	2.3

Initial rates of phosphorylation were determined by a continuous spectrophotomeric assay. The substituted amino acid residues in the analogs of YGEF peptide are underlined. The complete peptide sequences are given in Table 1.

an optimal Src phosphorylation sequence at these positions [7,22]. In terms of K_m and V_{max}, YGEF is the best substrate for wild-type v-Src (K_m = 98 μM and V_{max} = 120.3 μmol/min/mg) and for the L-472Y mutant (K_m = 65 μM and V_{max} = 3.4 μmol/min/mg). In contrast, the L-472W mutant phosphorylated the P+1 Ala peptide (YAEF) best with K_m = 100 μM and a V_{max}/K_m value of 30.0. The L-472W mutant also preferred YMEF (V_{max}/K_m = 23.6) over the parent YGEF peptide (V_{max}/K_m = 12.0). As seen in Table 2, these effects were most pronounced in the increases in V_{max} for the Ala/Met substitutions at the P+1 position. Thus, as observed in the initial screens (Fig. 3), the Leu-472 to Trp mutation in v-Src altered the preference at the P+1 position from Gly to Ala or Met. The T-429M mutant showed a low K_m value (53 μM) and high V_{max} (1.8 μmol/min/mg) towards YGEF and substitution of Gly with Ala in peptide YAEF resulted in a significant increase in K_m (470 μM). For the T-429M mutation in v-Src, the presence of a Thr residue at the P+3 position did not decrease V_{max}/K_m as dramatically as for wild-type v-Src and the other mutants (Table 2).

Overall, in terms of K_m and V_{max}/Km, YGEF is the best substrate for wild-type as well as for the L-472Y and T-429M mutants (V_{max}/K_m = 1227, 52.3 and 33.9 for wild-type, L-472Y and T-429M, respectively), suggesting that Gly at the P+1 position of YGEF peptides participates in a specific interaction between the substrate and v-Src. The exception to this overall specificity was the L-472W mutant form of Src. The L-472W mutant showed a preference for Ala or Met at the P+1 position in peptide substrates. Based on results for v-Abl [13], we expected that the L-472W mutation would primarily affect P+3 substrate recognition. A Trp mutation in the corresponding position of Abl completely altered P+3 substrate recognition towards synthetic peptides as well as towards Crk, a protein substrate possessing the ideal Abl recognition sequence. Thus, while this region of the Src and Abl catalytic domains may be involved in recognition of the P+1/P+3 positions, the precise contributions of different enzyme residues may vary between the two tyrosine kinases.

It is increasingly clear that the in vivo substrate specificity of a Src-family tyrosine kinase reflects not only the intrinsic specificity of the kinase catalytic domain, but also the effective local concentrations of protein substrates. The distribution of potential substrates is influenced by (a) the cellular distribution of enzyme and substrates and (b) interactions with non-catalytic regions of the enzymes, particularly the SH2 and SH3 domains [1]. These interactions recruit substrates to the vicinity of the tyrosine kinase catalytic domain. The question then arises, to what extent does the specificity of the catalytic domain of a tyrosine kinase govern specific signalling? An extreme view would be that the catalytic domains are non-specific, phosphorylating any protein that is presented to them by their associated SH2/SH3 domains. Evidence supporting this idea comes from experiments on Abl. In these studies, altering the Abl SH2 domain resulted in phosphorylation of alternative substrates in vivo [8], suggesting that interactions with non-catalytic domains play an important role in substrate specificity. On the other hand, the catalytic domains of tyrosine kinases have been demonstrated to have intrinsic specificities [7,14,22]. In at least one case, the intrinsic specificity of a tyrosine kinase catalytic domain has been shown to be important in signalling: the RET receptor tyrosine kinase [7,11,12]. Mutants with an altered catalytic domain specificity will be valuable tools in addressing the relative importance of individual domains in Src kinase signalling specificity.

Acknowledgements: This study was supported by National Institutes of Health Grant CA58530 (to W. Todd Miller).

References

[1] Brown, M.T. and Cooper, J.A. (1996) Biochim. Biophys. Acta 1287, 121–149.
[2] Pawson, T. (1995) Nature 373, 573–580.
[3] Cooper, J.A. and Howell, B. (1993) Cell 93, 1051–1054.
[4] Xu, W., Harrison, S.C. and Eck, M.J. (1997) Nature 385, 595–609.
[5] Sicheri, F., Moarefi, I. and Kuriyan, J. (1997) Nature 385, 602–609.
[6] Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C.-H., Kuriyan, J. and Miller, W.T. (1997) Nature 385, 650–653.
[7] Songyang, Z., Carraway, K.L., Eck III, M.J., Harrison, S.C., Feldman, R.A., Mohammadi, M., Schlessinger, J., Hubbard, S.R., Smith, D.P., Eng, C., Lorenzo, M.J., Ponder, B.A.J., Mayer, B.J. and Cantley, L.C. (1995) Nature 373, 536–539.
[8] Pellicena, P., Stowell, K.R. and Miller, W.T. (1998) J. Biol. Chem. 273, 15325–15328.
[9] Mayer, B.J. and Baltimore, D. (1994) Mol. Cell. Biol. 14, 2883–2894.
[10] Hubbard, S.R. (1997) EMBO J. 16, 5572–5581.
[11] Carlson, K.M., Dou, S., Chi, D., Scavarda, N., Toshima, K., Jackson, C.E., Wells, S.A., Goodfellow Jr., P.J. and Donis-Keller, H. (1994) Proc. Natl. Acad. Sci. USA 91, 1579–1583.
[12] Hofstra, R.M., Landsvater, R.M., Ceccherini, I., Stulp, R.P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J.W., van Amstel, H.K.P., Romeo, G., Lips, C.J.M. and Buys, C.H.C.M. (1994) Nature 367, 375–376.

[13] Till, J.H., Chan, P.M. and Miller, W.T. (1999) J. Biol. Chem. 274, 4995–5003.
[14] Garcia, P., Shoelson, S.E., George, S.T., Hinds, D.A., Goldberg, A.R. and Miller, W.T. (1993) J. Biol. Chem. 268, 25146–25151.
[15] Xu, B. and Miller, W.T. (1996) Mol. Cell. Biochem. 158, 57–63.
[16] Atherton, E. and Sheppard, R.C. (1989) in: Solid-Phase Peptide Synthesis: A Practical Approuch, IRL Press at Oxford University Press, Oxford.
[17] Casnellie, I.E. (1991) Methods Enzymol. 200, 115–120.
[18] Boerner, R.J., Barker, S.C. and Knight, W.B. (1995) Biochemistry 34, 16419–16423.
[19] Kraulis, P.J. (1991) J. Appl. Cryst. 24, 946–950.
[20] Yenush, L. and White, M. (1997) BioEssays 19, 491–500.
[21] Shoelson, S.E., Chatterjee, S., Chaudhuri, M. and White, M.F. (1992) Proc. Natl. Acad. Sci. USA 89, 2027–2031.
[22] Till, J.H., Annan, R.S., Carr, S.A. and Miller, W.T. (1994) J. Biol. Chem. 269, 7423–7428.