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Temperature sensitivities of metazoan and pre-metazoan Src kinases

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ABSTRACT

Homologous enzymes from different species display functional characteristics that correlate with the physiological and environmental temperatures encountered by the organisms. In this study, we have investigated the temperature sensitivity of the nonreceptor tyrosine kinase Src. We compared the temperature dependencies of c-Src and two Src kinases from single-celled eukaryotes, the choanoflagellate *Monosiga brevicollis* and the filasterean *Capsaspora owczarzaki*. Metazoan c-Src exhibits temperature sensitivity, with high activity at 30 °C and 37 °C. This sensitivity is driven by changes in substrate binding as well as maximal velocity, and it is dependent on the amino acid sequence surrounding tyrosine in the substrate. When tested with a peptide that displays temperature-dependent phosphorylation by c-Src, the enzymatic rates for the unicellular Src kinases show much less variation over the temperatures tested. The data demonstrate that unicellular Src kinases are temperature compensated relative to metazoan c-Src, consistent with an evolutionary adaptation to their environments.

1. Introduction

Temperature plays a key role in the adaptation of organisms to their environments. This is manifested in many aspects of physiology, including at the molecular level [1–3]. Elemental chemical processes are highly sensitive to temperature. The rates of most enzyme-catalyzed reactions would therefore be expected to increase 2- to 3-fold with each 10 °C increase in temperature, up to the point where protein three-dimensional structure is compromised [2]. In organisms that are adapted to colder environmental conditions, however, there are compensatory mechanisms to maintain enzymatic function. In such organisms, metabolic enzymes and other cellular proteins possess maximal activities at temperatures that are appropriate to the environment. These temperature-compensatory changes in enzyme function link cellular and whole organism temperature sensitivities.

Studies of the metabolic enzymes lactate dehydrogenase (LDH) and cytosolic malate dehydrogenase (MDH) have been particularly informative in understanding the molecular basis for cold adaptation. Compensation to temperature can involve changes in enzymatic reaction rates (i.e., k_{cat} effects) as well as changes in the binding of substrates (i.e., K_m effects) [4]. For example, LDH orthologs from Antarctic fish species (notothenoids) display a higher value of K_m (pyruvate) than related species living in temperate conditions, allowing pyruvate binding at the lower temperatures [5]. The notothenoid LDH enzymes also possess higher k_{cat} values than orthologs from warm-adapted animals [5]. At the protein structural level, two amino acid substitutions in notothenoid LDH are responsible for temperature compensation [6]. The amino acids occur in conformationally flexible loop regions of LDH that undergo movement during catalysis. Recently, studies of adenylate kinase have shown how adaptive changes on the surface of an enzyme can be coupled allosterically to the active site [7].

The circadian clock is another example of a physiological process that is temperature compensated [8]. The mechanism involves substrate and product binding by a specific domain of casein kinase 1δ, an important circadian period-regulatory kinase [9,10]. Temperature compensation was observed in the phosphorylation of small synthetic peptides by casein kinase 1δ, allowing the authors to determine kinetic parameters for Ser/Thr phosphorylation. Interestingly, temperature compensation was dependent on the sequence context [10]. For many other signaling kinases, and in particular for tyrosine kinases, it is not known how activity depends on temperature, or whether activity towards particular substrates might be affected.

c-Src is a nonreceptor 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 [11,12]. c-Src is a member of a family of nine related nonreceptor tyrosine kinases, collectively called Src family kinases (SFKs). v-Src, the viral homolog of c-Src, is the product of the transforming gene from Rous sarcoma virus [13,14]. SFKs possess an

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Abbreviations

ATP	adenosine triphosphate
Fmoc	fluorenylmethoxycarbonyl
IGF1R	insulin-like growth factor 1 receptor
LDH	lactate dehydrogenase
MDH	malate dehydrogenase
PMSF	phenylmethylsulfonyl fluoride
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
YAEF	peptide AEEEIYAEFEAKKKG
YGEA	peptide AEEEIYGEAEAKKKG
YGEF	peptide AEEEIYGEFEAKKKG

evolutionarily conserved domain arrangement consisting 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 involved in the negative regulation of kinase activity [15,16].

The components of phosphotyrosine (pTyr)-based signal transduction (receptor and nonreceptor tyrosine kinases, pTyr-binding domains, and tyrosine phosphatases) have been identified in choanoflagellates, the closest living relatives to metazoans, as well as in other single-celled protists. The genomes of these premetazoans contain orthologs of mammalian c-Src, as well as homologs of Csk, Abl, and Tec nonreceptor kinases [17–20]. The SH3-SH2-kinase arrangement of signaling domains in SFKs, which is essential for activity and specificity [15,21], is strictly conserved across all species. In this study, because mammals and protists occupy widely divergent environmental niches, we tested the temperature sensitivities of metazoan and protist Src kinases. We report a degree of temperature sensitivity that is substrate-dependent and is compensated in the premetazoan Src orthologs.

2. Materials and methods

2.1. Materials

Nickel-nitriloacetic acid resin was purchased from Qiagen. Leupeptin, aprotinin, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), and sodium orthovanadate were from Sigma. The Sf9 insect cells were maintained in Sf-900 medium (Gibco) supplemented with 5% fetal bovine serum and antibiotic/antimycotic.

2.2. Protein expression and purification

All tyrosine kinase constructs included SH3, SH2, and kinase domains, as well as the C-terminal regulatory tail sequences. c-Src kinase was a kind gift from Dr. Markus Seeliger (Stony Brook). MbSrc1 and CoSrc2 were expressed in *Spodoptera frugiperda* (Sf9) insect cells using the Bac-to-Bac system (Invitrogen), and purified as described previously [22,23]. Briefly, Sf9 cells (800 ml) were infected with recombinant baculovirus and harvested after three days. Cells were lysed in a French pressure cell, and His-tagged kinases were purified using nickel-nitrilotriacetic acid resin. Peak fractions were pooled and concentrated in an Amicon Ultrafiltration device (molecular weight cutoff: 30,000 Da). The purified proteins were stored in 40% glycerol at −20 °C.

2.3. Kinase assays

Kinase assays were performed using the phosphocellulose paper binding assay [24]. Reaction mixtures (25 μl) contained 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.2 mM ATP, [γ-^{32}P]-ATP (30–50 cpm/pmol), and varying amounts of kinase and peptide substrates. The reaction mixtures were pre-incubated at the indicated times for 15 min in temperature-controlled water baths. Reactions were initiated by addition of enzyme, and were maintained at the indicated temperatures. The reactions were quenched by the addition of 45 μl 10% trichloroacetic acid. After centrifugation, supernatants were spotted on phosphocellulose paper and washed three times with 0.5% phosphoric acid. Incorporation of ^{32}P into peptide was determined by liquid scintillation counting.

All peptide substrates were prepared by solid phase peptide synthesis on an Applied Biosystems automated peptide synthesizer using standard Fmoc chemistry. The peptides were purified by reverse-phase high pressure liquid chromatography, and characterized by mass spectrometry prior to use. The following substrates were used: AEEEIYGEFEAKKKG, AEEEIYGEAEAKKKG, and AEEEIYAEFEAKKKG. For determination of K_m (peptide) values, the enzyme concentrations were 192 nM and the peptide concentrations ranged from 0-1000 μM. Kinetic constants were determined by nonlinear fitting to the [substrate] vs. velocity curves using GraphPad Prism 8.

2.4. Molecular modeling

The three-dimensional structure of MbSrc1 was modeled on the known structure of chicken c-Src (pdb: 6HVE) using the homology detection program HHPred and MODELLER v. 9.22 from the MPI Bioinformatics Toolkit [25,26].

3. Results

3.1. Temperature dependence of Src activity

We previously showed that a synthetic peptide with the sequence AEEEIYGEAEAKKKG (denoted YGEA in this study) was an efficient substrate for the 60-kDa Rous sarcoma virus v-Src kinase [27]. Here, we tested this substrate with a purified c-Src kinase construct. The metazoan c-Src used in this study possessed the chicken c-Src sequence. (Chicken c-Src catalytic domain is identical to the human sequence, except for a single glutamate to aspartate substitution). Chicken c-Src was the initial cellular form of Src to be characterized [28], and the majority of studies of Src regulation and substrate specificity have used this enzyme [15]. A three-dimensional structure of the enzyme is also available [29]. We carried out initial experiments at different temperatures by pre-incubating reaction mixtures containing peptide at 4 °C, 18 °C, 30 °C, or 37 °C for 15 min prior to addition of enzyme. The reactions proceeded for 10 min at the indicated temperatures. The reactions with peptide YGEA showed a pronounced temperature dependence, with maximum Src activity at 30 °C or 37 °C (Fig. 1).

The rates of most enzymatic reactions increase by 2- to 3-fold with each 10 °C increase in temperature, yielding the "Q_{10} relationship" of thermal physiology [2]. We selected 18 °C and 30 °C to investigate temperature dependence further, because this 12-degree difference resulted in a large change in c-Src activity (Fig. 1). We measured initial rates of peptide YGEA phosphorylation at the two temperatures (Fig. 2). The initial rate at 30 °C was 5.68 times higher than the rate at 18 °C (i.e., $Q_{12} = 5.68$).

The sensitivity of enzymes to temperature can be dictated by substrate or ligand binding affinity or by catalytic rate [4]. To distinguish between these possibilities, we carried out steady-state kinetic assays at 18 °C and 30 °C using variable concentrations of peptide YGEA substrate (Fig. 3). These experiments showed that the peptide K_m at 30 °C was 1.9-fold lower than at 18 °C, while the V_{max} was 4.6-fold higher (Table 1). A combination of effects on substrate binding and catalysis therefore appears to underlie the temperature sensitivity of c-Src towards peptide YGEA.

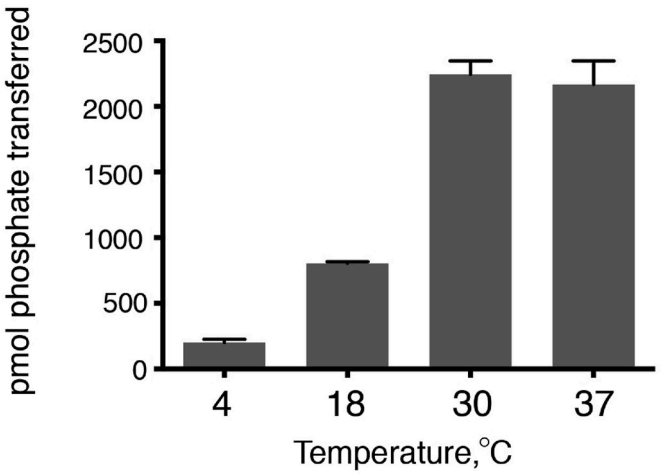

Fig. 1. Temperature dependence of c-Src. Duplicate reactions containing 1 mM peptide YGEA were preincubated for 15 min at 4 °C, 18 °C, 30 °C, or 37 °C. After addition of enzyme (192 nM), reactions proceeded for 10 min at the indicated temperatures, and peptide phosphorylation was measured by the phosphocellulose paper binding assay. This experiment was repeated three times with similar results.

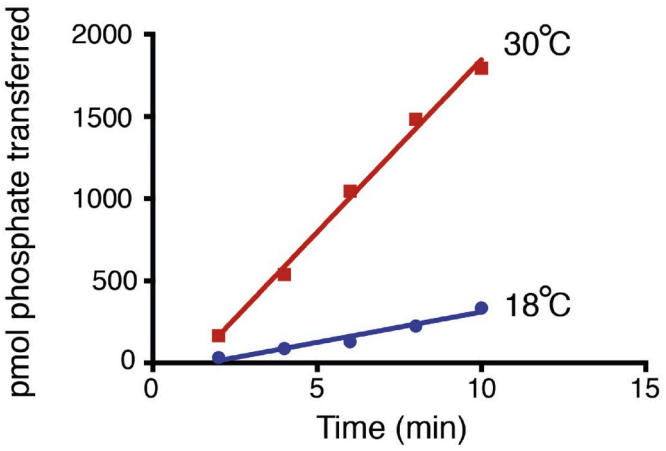

Fig. 2. Initial rates of YGEA phosphorylation by c-Src at 18 °C (blue) and 30 °C (red). The conditions were similar to those used in the legend to Fig. 1. Reactions were stopped at the indicated times. The enzyme concentration was 160 nM. The lines show linear fits to the data. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Temperature dependence depends on peptide sequence

The degree of temperature compensation for a particular enzyme can be dependent on the identity of the substrate. For example, in a recent study of casein kinase 1δ-dependent phosphorylation, it was demonstrated that temperature sensitivity was greatly affected by the peptide sequence of the target peptide [10]. We tested c-Src activity towards the closely related peptide substrate AEEEIYGEFEAKKKKG [30] (YGEF), containing a single amino acid substitution (Phe replacing Ala) at the P+3 position relative to the phosphorylatable tyrosine. Phosphorylation of this peptide showed a markedly decreased variance over the range of temperatures (a phenomenon known as temperature compensation [2,3]) (Fig. 4). To quantify the rate differences at 18 °C and 30 °C, we measured the initial rates of peptide YGEF phosphorylation. The rates at 18 °C and 30 °C were very similar, with $Q_{12} = 1.09$ (Table 2). Next, we measured c-Src activity at various temperatures toward the peptide AEEEIYAEFEAKKKKG; this peptide (YAEF) also has Phe at the P+3 position, but contains Ala rather than Gly at the P+1 position [27]. This substrate was also temperature compensated relative to peptide YGEA (Fig. 4). The Q_{12} value for YAEF was 0.93 (Table 2). These experiments identified the residue at the P+3 position of peptide substrate as a key

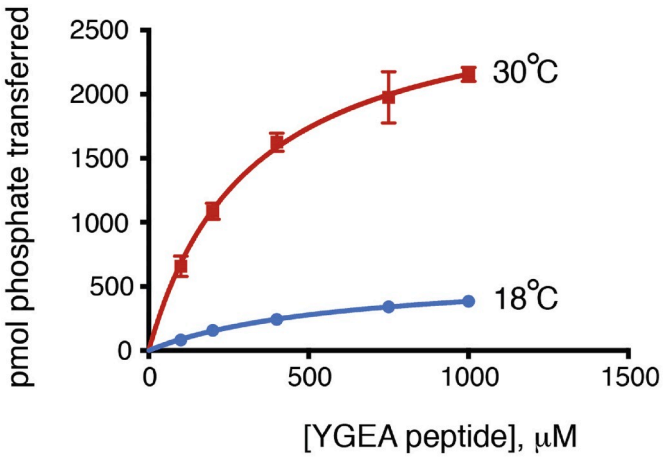

Fig. 3. c-Src temperature dependence at different concentrations of peptide YGEA. Initial rates of phosphorylation were measured at 18 °C (blue) and 30 °C (red) with varying (100 μM-1mM) concentrations of peptide YGEA. Values shown are ± standard deviation. The enzyme concentration was 192 nM. The lines show nonlinear fits to the hyperbolic velocity vs. [substrate] curves. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Kinetic parameters for c-Src phosphorylation of peptide YGEA at 18 °C and 30 °C.

	18 °C	30 °C
K_m (peptide), μM	607.6 ± 89	319.1 ± 40.5
V_{max}, pmol/min	77 ± 5.5	355.8 ± 17.1

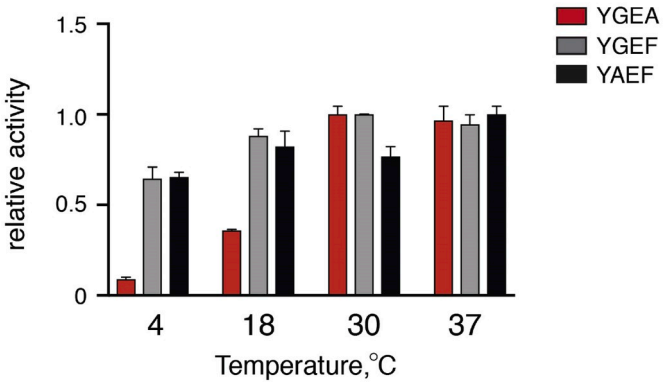

Fig. 4. Temperature dependence of c-Src toward three peptide substrates. The data from Fig. 1 for YGEA (red) are shown in comparison to data from reactions with YGEF (gray) and YAEF (black). Peptide concentrations were 1 mM and enzyme concentration was 192 nM. The data are presented as activities relative to the maximum for each peptide. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Q_{12} values (ratios of initial rates of c-Src phosphorylation at 30 °C to rates at 18 °C).

	YGEA	YGEF	YAEF
Q_{12} value	5.68	1.09	0.93

determinant of temperature sensitivity.

3.3. Temperature compensation in unicellular c-Src kinases

Eukaryotic-like receptor and nonreceptor tyrosine kinases are absent in bacteria, plants, yeast, and other fungi. On the other hand, genomic analyses of several groups of unicellular eukaryotes have demonstrated the presence of numerous tyrosine kinases [18,31]. This was first noted through the study of the unicellular choanoflagellate *Monosiga brevicollis* [17,19]. Choanoflagellates are protists that live in fresh water or in the oceans, and they are considered to be the closest living single-celled relatives to metazoans (Fig. 5A) [32,33]. The kinome of *M. brevicollis* contains many nonreceptor tyrosine kinases that are close homologs of mammalian kinases, including c-Src and c-Abl [19,20]. MbSrc1, one of four *M. brevicollis* Src kinases, shares many features with mammalian c-Src; it has the conserved SH3-SH2-kinase modular structure, it has a similar substrate specificity, and it is activated by autophosphorylation within the activation loop [22]. Because thermal adaptation is one mechanism by which organisms are adapted to their environmental niches, we tested the temperature sensitivity of purified MbSrc1 kinase. The activity of MbSrc1 was much less sensitive to temperature than that of c-Src, as measured by phosphorylation of the YGEA peptide (Fig. 5B). Even at 4 °C, MbSrc1 maintained significant activity, corresponding to roughly half that of its maximum (Fig. 5B). A comparison of the rates at 18 °C and 30 °C (165.4 pmol/min and 172.8 pmol/min, respectively) gave a Q_{12} value of 1.05 (data not shown).

We carried out similar experiments on purified CoSrc2, one of the two Src-like kinases from the filasterean *Capsaspora owczarzaki* [23]. Filastereans represent a sister group to metazoans and choanoflagellates (Fig. 5A), and the kinome of *C. owczarzaki* contains a large and diverse group of tyrosine kinases [34]. Like MbSrc1, CoSrc2 contains SH3, SH2, and kinase domains in a similar arrangement as mammalian Src family kinases, and it is active towards many of the same substrates *in vitro* [23]. CoSrc2 showed activity across all temperatures, including 4 °C (Fig. 5B). In contrast to MbSrc1, CoSrc2 showed some temperature dependence towards the YGEA peptide. The rates at 18 °C and 30 °C were 73.8 pmol/min and 114.3 pmol/min, respectively (Q_{12} = 1.55; data not shown). Thus, the difference between 18 °C and 30 °C was much smaller for CoSrc2 than for c-Src. Collectively, the data demonstrate that unicellular Src kinases are temperature compensated relative to metazoan c-Src.

4. Discussion

Orthologous proteins from organisms inhabiting divergent environments frequently show evidence of adaptation. Previous work has uncovered numerous examples of functional differences between enzymes from differently thermally adapted species [2,4]. In some cases, the molecular basis for adaptation has also been deduced. Here, we have investigated the temperature sensitivity of multiple forms of a tyrosine kinase, namely the nonreceptor tyrosine kinase Src. We have compared a metazoan c-Src with two unicellular forms of the enzyme. While it was originally believed that eukaryotic-like tyrosine kinases would only be present in multicellular animals, recent studies have shown that several groups of unicellular protists contain abundant numbers of tyrosine kinases. Choanoflagellates and filastereans, the groups whose Src kinases were studied here, are of interest because their phylogenetic positions provide important information about the origins of animal multicellularity [31,33,35].

Choanoflagellates such as *M. brevicollis* are widely distributed in both marine and freshwater environments [35,36]. While most have been isolated from water depths of 0–25 m, choanoflagellates have been found at depths of up to 300 m, and they have been isolated from the Arctic region as well as from warmer oceans [37]. *M. brevicollis* has found wide use for the study of choanoflagellates because it grows readily in the laboratory under a variety of conditions [38], and it possesses both sedentary and motile stages, making it an interesting model for investigating life cycle transitions [39]. The filasterean *C. owczarzaki* was originally isolated as an amoeba-like symbiont of a fresh-water snail sampled in Puerto Rico [40]. The temperature sensitivity of *C. owczarzaki* has not been studied thoroughly, but a recent report indicates that this filasterean is viable and can be transfected with heterologous DNA at 18 °C [41].

We found that c-Src exhibits temperature sensitivity, with maximum activity at 30 °C and 37 °C (Figs. 1–3). As was the case for the metabolic enzyme lactate dehydrogenase [5], this temperature sensitivity appears to depend on substrate binding (effects on K_m for peptide substrate) as well as the rate-limiting chemical/conformational steps of catalysis (effects on k_{cat}) (Fig. 3 and Table 1). While these two effects counterbalance each other in some enzymes [10], for c-Src the lower K_m and

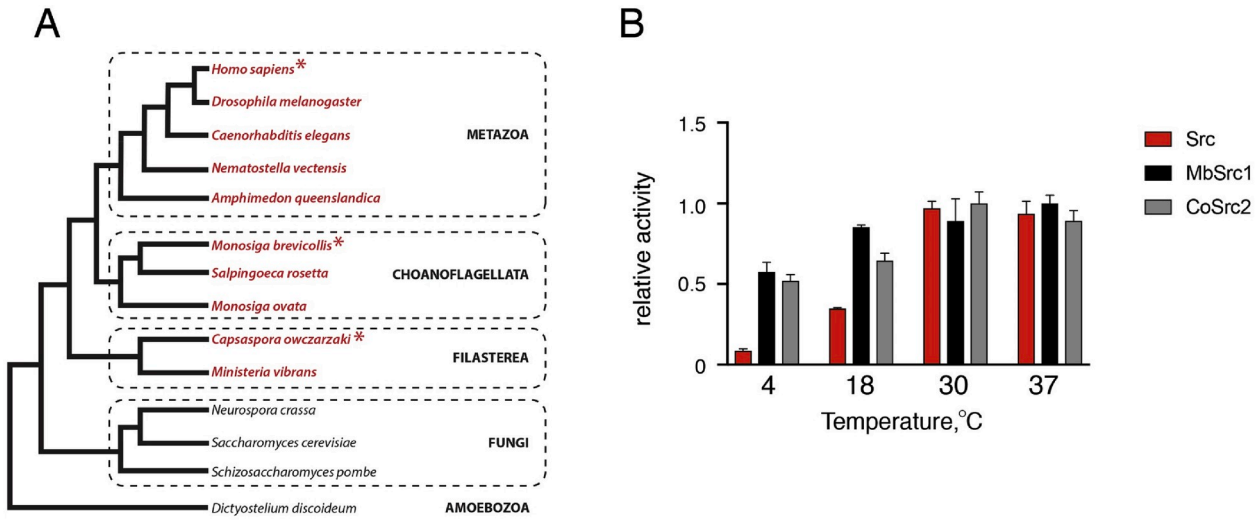

Fig. 5. (A). Distribution of tyrosine kinases. The eukaryotic tree of life is shown schematically. Species containing eukaryotic-like tyrosine kinases are shown in red. Species marked with a red asterisk (*) represent Src kinases studied in this report. **(B).** Temperature dependence of c-Src compared to unicellular Src kinases. Human c-Src (red), *Monosiga brevicollis* MbSrc1 (black), or *Capsaspora owczarzaki* CoSrc2 (gray) were assayed with 1 mM YGEA at the indicated temperatures using the phosphocellulose paper binding assay. All enzyme concentrations were 192 nM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher k_{cat} at 30 °C (as compared to 18 °C) combine to produce a strong overall effect on k_{cat}/K_m.

Interestingly, the strong degree of temperature sensitivity observed for c-Src (Q_{12} for YGEA = 5.68) was peptide substrate dependent (Fig. 4). This is similar to the results obtained for casein kinase 1δ, where temperature dependency of phosphorylation was affected by the target peptide sequence context. Phosphorylation of casein itself by casein kinase 1δ was sensitive to temperature, while phosphorylation of peptides derived from the substrates βTrCP and FASPS showed temperature compensation [42]. The determinants for temperature compensation were identified by studies of peptide variants [10]. In a similar fashion, we studied three closely related synthetic peptides for c-Src, and found temperature compensation in peptides YAEF and YGEF, with Q_{12} values close to 1.0. The results of these studies point to the amino acid residue at the P+3 position (relative to Tyr) as being a critical determinant for temperature compensation.

The unicellular forms of Src kinase studied here consist of the same domain arrangement as c-Src [15], and share many of the enzymatic and regulatory properties of their metazoan counterpart [22,23]. Nonetheless, we observed strong temperature compensation in these enzymes relative to c-Src (Q_{12} = 1.05 and 1.55 for MbSrc and CoSrc2 for the YGEA peptide, respectively). This is consistent with the hypothesis that Src kinases are thermally adapted for their environmental niches. One plausible model is that the last common ancestor between unicellular and multicellular organisms was temperature insensitive, and that sensitivity was gained through evolution. Additionally or alternatively, the premetazoan forms of Src may have a more relaxed substrate preference at the P+3 position. These possibilities will be clarified when more information is available about the physiological substrates of these enyzmes.

To study the structural basis for temperature compensation in MbSrc1, a first step would be to identify residues that interact with the P+3 position of peptide substrates. There are relatively few cocrystal structures of tyrosine kinases with bound peptide substrates, and there are currently no such direct structural data for a Src-peptide complex. The kinase residues that interact with bound substrate have been identified for insulin and IGF1R receptor kinases [43,44]. For IGF1R, residues in the αEF and β11 regions of the kinase C-terminal lobe, particularly L1144, M1149, L1154, and G1157, make contact with residues at the P+3 to P+5 positions of the substrate [43]. The corresponding region in Src kinases is mostly conserved between chicken and premetazoan Src kinases, with the exception of a Gly residue at the position corresponding to IGF1R G1157 (G437 in chicken Src, within the sequence YGRFT) (Fig. 6A). The corresponding residue is Asn in both MbSrc1 (N399) and CoSrc2 (N492). MbSrc1 contains several non-conserved amino acids within this motif (sequence: MNRFS). The corresponding sequence in CoSrc2 is YNRFS (Fig. 6A). Thus, this region of MbSrc1 (residues 398–402) is a possible contributor to temperature compensation. We created a molecular model of MbSrc1 based on the three-dimensional structure of chicken c-Src (Fig. 6B), and highlighted the position of N399 within the putative peptide binding site. An alternative view of the model is shown in Supplemental Fig. S1, with the entire activation loop depicted in spheres.

On the other hand, a recent NMR and molecular dynamics study of c-Src identified a different group of residues in the Src P+1 loop that contribute to peptide substrate binding [45]. In MbSrc1, E382 is present at the position corresponding to one such residue in c-Src (Q420 of chicken c-Src) (Fig. 6). The residue in CoSrc2 at that position is also a glutamate (E475), in contrast to human, chicken, fly, worm, and fish Src kinases, which do not have an acidic residue at that position (Fig. 6A). This residue and the surrounding sequence could also play a role in temperature compensation by the unicellular enzymes. Future experimental manipulation of these two regions of c-Src, MbSrc1, and CoSrc2 should help to define the basis for temperature sensitivity and compensation in these signaling enzymes.

5. Conclusions

The kinase activity of a metazoan c-Src shows a dependence on temperature. This temperature sensitivity depends on the sequence of the peptide substrate; the identity of the amino acid at the P+3 position (relative to the phosphorylatable tyrosine) appears to play a particularly important role. The activities of homologous Src kinases from the single-celled protists *M. brevicollis* and *C. owczarzaki* show a lower degree of variation with temperature with one substrate peptide, consistent with the hypothesis that Src kinases are thermally adapted for their environmental niches.

Author contributions

WTM conducted the experiments and wrote the manuscript.

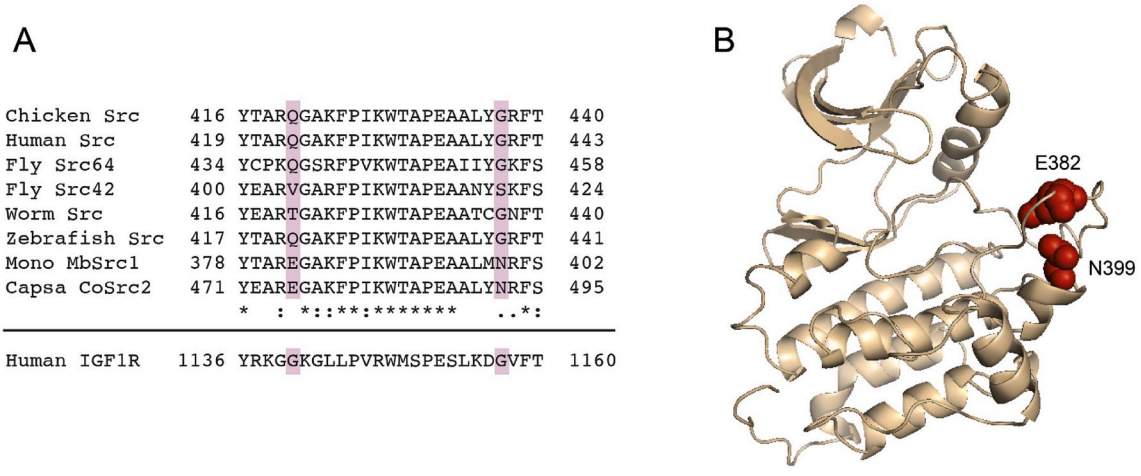

Fig. 6. (A) Alignment of selected Src kinases. The alignment was performed with Clustal Omega (clustal.org) using the sequences of chicken and human c-Src; *D. melanogaster* Src42A and Src64B; *C. elegans* Src-1; *D. rerio* Src; *M. brevicollis* MbSrc1; and *C. owczarzaki* CoSrc2. Shaded residues are divergent between premetazoan and metazoan Src kinases, and could potentially interact with peptide substrates (see text for details). Asterisks: conserved residues; colons: strongly similar; periods: weakly similar. The homologous region of human IGF1R is shown below the line. (B) Homology model of the MbSrc1 catalytic domain, based on the three-dimensional structure of chicken c-Src (pdb code 6HVE). MbSrc1 residues that could potentially interact with C-terminal residues of peptide substrates are shown as spheres and are colored red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100775

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100775.

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