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., $k_{\text{cat}}$ 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 $k_{\text{cat}}$ 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 16, an important circadian period-regulatory kinase [9,10]. Temperature compensation was observed in the phosphorylation of small synthetic peptides by casein kinase 16, 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
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 protists contain orthologs of mammalian c-Src, as well as homologs of Csk, Abl, and Tec nonreceptor kinases [17–20]. The SH3-SH2-kine 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-nitrosoacetic 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 (Spf9) 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-nitrotriacetic 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 MgCl2, 0.2 mM ATP, [γ-32P]-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 32P 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: AEEEIYAGEEAKKKKG, AEEEIYGEAEAKKKKG, and AEEEIYGEAEAKKKKG. For determination of Kcat (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 AEEEIYGEAEEKKKKG (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 enzymatic reactions increase by 2- to 3-fold with each 10°C increase in temperature, yielding the “Q10” 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., Q12 = 5.68).

The sensitivity of enzymes to temperature can be dictated by substrate or ligand binding affinity or by catalytic rate [3]. 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 Km at 30°C was 1.9-fold lower than at 18°C, while the Vmax 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.
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 Q12 = 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 Q12 value for YAEF was 0.93 (Table 2). These experiments identified the residue at the P+3 position of peptide substrate as a key position, but does not affect the temperature compensation.}

Table 1

|            | 18 °C   | 30 °C   |
|------------|---------|---------|
| Km (peptide), μM | 607.6 ± 89 | 319.1 ± 40.5 |
| Vmax, pmol/min  | 77 ± 5.5  | 355.8 ± 17.1  |

Table 2

| peptide | Q12 value |
|---------|-----------|
| YGEA    | 5.68      |
| YGEF    | 1.09      |
| YAEF    | 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 metazoa (Fig. 5A) \[22,23\]. The genome 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 Q12 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 \[23\] (Fig. 5A). 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 (Q12 = 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 freshwater 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 *Km* for peptide substrate) as well as the rate-limiting chemical/conformational steps of catalysis (effects on *kcat*) (Fig. 3 and Table 1). While these two effects counterbalance each other in some enzymes \[10\], for c-Src the lower *Km* and...
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.)

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 (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.
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