Early emergence of negative regulation of the tyrosine kinase Src by the C-terminal Src kinase

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Stringent regulation of tyrosine kinase activity is essential for normal cellular function. In humans, the tyrosine kinase Src is inhibited via phosphorylation of its C-terminal tail by another kinase, C-terminal Src kinase (Csk). Although Src and Csk orthologs are present across holozoan organisms, including animals and protists, the Csk–Src negative regulatory mechanism appears to have evolved gradually. For example, in choanoflagellates, Src and Csk are both active, but the negative regulatory mechanism is reportedly absent. In filastereans, a protist clade closely related to choanoflagellates, Src is active, but Csk is apparently inactive. In this study, we use a combination of bioinformatics, in vitro kinase assays, and yeast-based growth assays to characterize holozoan Src and Csk orthologs. We show that, despite appreciable differences in domain architecture, Csk from Corallochytrium limacisporum, a highly diverged holozoan marine protist, is active and can inhibit Src. However, in comparison with other Csk orthologs, Corallochytrium Csk displays broad substrate specificity and inhibits Src in an activity-independent manner. Furthermore, in contrast to previous studies, we show that Csk from the filasterean Capsaspora owczarzaki is active and that the Csk–Src negative regulatory mechanism is present in Csk and Src proteins from C. owczarzaki and the choanoflagellate Monosiga brevicollis. Our results suggest that negative regulation of Src by Csk is more ancient than previously thought and that it might be conserved across all holozoan species.

Eukaryotic protein–tyrosine kinases transmit cellular signals by phosphorylating tyrosine residues in proteins (1, 2). Tyrosine kinases are structurally similar to serine/threonine kinases, which phosphorylate proteins on serine and threonine residues (3, 4). Although serine/threonine kinases are present in all eukaryotes, tyrosine kinases are only found in metazoans and their close unicellular relatives (5–7). Tyrosine kinases are thus believed to have played an essential role in the evolution of metazoan multicellularity, offering a new mechanism for signal transduction and allowing for development of the more complex signaling networks found in metazoans (6, 7).

Analyses of holozoan genomes revealed that tyrosine kinases have undergone extensive branch-specific diversification and gene loss (3, 8–13). Nevertheless, a core set of tyrosine kinases is found in most holozoans (14). Two cytoplasmic tyrosine kinases included in this core set are Src and the C-terminal Src kinase (Csk).3 Src plays an important regulatory role in a variety of cellular functions, and aberrant Src activity is linked to cancer (15, 16). In humans, Src activity is inhibited by Csk, which phosphorylates residue Tyr-530 in the C-terminal tail of Src (17). This phosphorylation drives binding of the C-terminal tail of Src by its SH2 domain, resulting in a closed, inactive conformation (18, 19).

Biochemical and mammalian cell-based analyses of Src and Csk from a variety of organisms have shown that the Csk–Src negative regulatory mechanism is present in early metazoans, like hydra and sponge, but weak or absent in non-metazoans, like choanoflagellates and filastereans (Fig. 1) (20–24). For example, although Csk can phosphorylate Src at the C-terminal tail regulatory tyrosine in the choanoflagellates Monosiga brevicollis and Monosiga ovata, phosphorylation apparently does not lead to inactivation of Src (21, 22). Additionally, Csk from the filastereans Capsaspora owczarzaki and Ministeria vibrans is reportedly inactive in vitro and in mammalian cellular assays (23, 24). These findings led to the hypothesis that negative regulation of Src by Csk is specific to Metazoa, with the absence of the negative regulatory mechanism in other clades being attributed to structural constraints in the Src kinase domain and Src interdomain contacts (21–24). Furthermore, some have suggested that Csk either evolved as a pseudokinase or that Csk activity was lost in the filasterean lineage (23, 24).

However, some questions remain. Does the Csk–Src negative regulatory mechanism appear only in Metazoa? Or could the negative regulation have appeared early and then been subsequently lost in Choanoflagellata and Filasterea? The recent discovery of Src- and Csk-encoding genes in Ichthyosporea and Corallochytrea (12), two holozoan groups that are less closely related to Metazoa than are Choanoflagellata and Filasterea (25, 26), create the opportunity to answer these questions. Thus, we characterized Src and Csk from Corallochytrium

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This article contains supplemental Figs. S1–S7 and Tables S1–S3.

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3 The abbreviations used are: Csk, C-terminal Src kinase; SH, Src homology; KD, kinase-dead; CHK, Csk-homologous kinase; LB, lysogeny broth.
Corallochytrium limacisporum using bioinformatic analysis, in vitro kinase assays, and yeast-based growth assays. We found that the domain architecture of Src from C. limacisporum is highly conserved, whereas C. limacisporum Csk contains several insertions and also lacks an SH3 domain. Despite this, Src and Csk from C. limacisporum are both active tyrosine kinases, and the Csk–Src negative regulatory mechanism is present in these early holozoan proteins. However, C. limacisporum Csk has a broad substrate specificity compared with other Csk proteins, a difference we attribute to its altered sequence and domain architecture. We also characterized Src and Csk from the choanoflagellate M. brevicollis and the filasterean C. owczarzaki and observed several differences from previous studies. For example, Csk from C. owczarzaki is an active tyrosine kinase. In fact, Src and Csk from both organisms are about as active as their human orthologs, and Src activity is inhibited by Csk in both cases. Our results suggest that negative regulation of Src by Csk is more ancient than previously thought and that it might be conserved across all holozoan species.

Results

Corallochytrium limacisporum Src and Csk retain key functional residues

Src and Csk orthologs in C. limacisporum, CiSrc and CiCsk, were found in transcriptome sequencing data (12). We compared CiSrc and CiCsk with other holozoan Src and Csk sequences using amino acid sequence alignments (Fig. 2A and supplemental Fig. S1). The kinase domains for both proteins are well conserved, with all catalytically important residues in place (Fig. 2A and supplemental Fig. S1). These include the three glycine residues that position ATP, the catalytic lysine residue in β-strand 3, the glutamate residue in the αC-helix that stabilizes the catalytic lysine, and the aspartic acid in the catalytic loop that acts as the catalytic base (3). Furthermore, the residues important for interaction between Csk and Src are conserved in CiSrc and CiCsk (27). These include five arginine residues in the C-lobe of the Csk kinase domain and both lysine 442 and aspartic acid 518 in Src.

A phylogenetic analysis suggested that both Src and Csk were present in the common ancestor of holozoans (supplemental Fig. S2). Although the domain architecture of CiSrc is well conserved, CiCsk differs from other Csk sequences in several ways. Most strikingly, CiCsk lacks an SH3 domain (Fig. 2B). Furthermore, CiCsk has insertions in the SH2–kinase domain linker and in the kinase domain between β-strands 2 and 3, between β-strands 7 and 8, and in the activation loop. CiCsk also has an extended C-terminal region consisting of ~50 residues. Any of these differences could significantly alter the activity or substrate specificity of CiCsk. However, despite these differences, the preservation of important sequence features suggested that CiCsk could be functional.

CiSrc and CiCsk are active protein–tyrosine kinases

To determine whether CiSrc and CiCsk are functional tyrosine kinases, we expressed them in Escherichia coli and then purified them. As has been done before for the human Src ortholog HsSrc, we expressed an N-terminally truncated form of CiSrc encompassing residues 80–528 (27, 28). Expression levels for both truncated CiSrc and full-length CiCsk were low, but co-expression with the phosphatase YopH and the chaperone GroEL yielded ~10 µg of CiSrc and ~2 µg of CiCsk per 1 ml of cell culture (supplemental Fig. S3A). In comparison,
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HsSrc and HsCsk, expressed in the absence of YopH and GroEL, yielded ~15 μg and ~30 μg protein per 1 ml of cell culture, respectively (supplemental Fig. S3B). The tyrosine kinase activity of each protein was assayed using a universal tyrosine kinase peptide substrate, poly(Glu_4,Tyr), in a luminescence-based kinase assay. As a control, we evaluated kinase-
dead mutants in which the catalytic lysine residue in β-strand 3 was mutated to methionine in ClSrc (ClSrc-KD) or to arginine in ClCsk (ClCsk-KD, supplemental Table S1).

We found that ClSrc tyrosine kinase activity was comparable with HsSrc (Fig. 3A). However, ClCsk displayed reduced phosphorylation activity relative to HsCsk (Fig. 3B). Lower activity of ClCsk relative to HsCsk might arise because ClCsk lacks an SH3 domain; HsCsk lacking its SH3 domain is 10-fold less active than full-length HsCsk (29). Other sequence differences, such as the insertions in the ClCsk kinase domain, might also reduce activity. Finally, the purification procedure or assay conditions we employed might have partially inactivated ClCsk. In any case, both ClSrc and ClCsk possess measurable tyrosine kinase activity in vitro.

To determine whether ClCsk might inhibit ClSrc, we measured the ability of ClCsk to phosphorylate the C-terminal tyrosine residue in ClSrc. We eliminated any confounding ATP consumption caused by ClSrc autophosphorylation by using a kinase-dead ClSrc mutant (ClSrc-KD, Fig. 3C). We found that ClCsk phosphorylates ClSrc-KD and that the level of ClSrc-KD phosphorylation by ClCsk is similar to the level of HsSrc-KD phosphorylation by HsCsk. As a negative control, we also mutated the C-terminal regulatory tyrosine residue of ClSrc-KD to phenylalanine (ClSrc-KD-tail). ClCsk does not phosphorylate ClSrc-KD-tail, indicating that ClCsk phosphorylates ClSrc in a specific manner. These results suggest that ClCsk could inhibit ClSrc via phosphorylation at the C-terminal tyrosine residue.

ClCsk can inhibit ClSrc activity in a yeast growth assay

Measuring negative regulation of ClSrc by ClCsk in the in vitro tyrosine kinase assay would have been difficult because both kinases would have simultaneously produced signal. Thus, we turned to a growth-based assay in Saccharomyces cerevisiae to measure negative regulation of Src by Csk (30). When expressed in yeast, HsSrc causes a profound growth defect that is presumably related to unopposed tyrosine phosphorylation of yeast proteins (Fig. 3D and supplemental Fig. S4). However, growth is not inhibited when a kinase-dead mutant of HsSrc, HsSrc-KD, is expressed instead. Co-expression of HsCsk rescues the HsSrc growth defect. Consistent with canonical HsCsk-mediated negative regulation of HsSrc, HsCsk is not able to rescue growth when the C-terminal regulatory tyrosine of HsSrc is mutated to a phenylalanine (HsSrc-tail). Furthermore, a kinase-dead HsCsk mutant (HsCsk-KD) is not able to rescue growth. As in the in vitro assay, we used the N-terminally truncated form of Src. Full-length HsSrc yielded similar results.
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(supplemental Fig. S5). Thus, the yeast assay provides a convenient method for evaluating the ability of Csk to inhibit Src.

As expected based on its high level of in vitro tyrosine kinase activity, ClSrc inhibited yeast growth about as strongly as HsSrc (Fig. 3D and supplemental Fig. S4). CICsk fully rescued the growth of yeast expressing ClSrc, showing that CICsk can inhibit ClSrc activity. Interestingly, kinase-dead CICsk (CICsk-KD) also fully rescued the growth of yeast expressing ClSrc. Thus, CICsk appears to inhibit ClSrc regardless of whether it is active. This apparent negative regulation of Src in the absence of Csk kinase activity has not been observed for any other Csk/Src pairs. However, the Csk-homologous kinase, CHK, inhibits the Src family kinase Hck by binding alone (31). The analogy to CHK is not perfect; unlike CICsk, kinase-dead CHK cannot inhibit Hck. Furthermore, CICsk does not inhibit the activity of ClSrc-tail, whereas CHK does inhibit Hck-tail (31). Based on these findings, we speculate that ClCsk might inhibit ClSrc by binding and that the ClSrc C-terminal regulatory tyrosine could play an important role in this interaction. Despite these complexities, our results suggest that the Csk–Src negative regulatory mechanism is intact in Csk and Src from *C. limacisporum.*

We quantified protein expression and tyrosine phosphorylation in yeast by Western blotting (supplemental Fig. S6, A and B). The expression level of different HsSrc and ClSrc mutants varied considerably. Here, active mutants of Src were lowly expressed, whereas inactive mutants of Src were highly expressed. This inverse dependence of Src expression on activity level has been observed previously in mammalian cells, where it was linked to ubiquitin-mediated Src degradation (32). In contrast, the expression level of different HsCsk and ClCsk mutants was uniformly high. HsCsk phosphorylates the C-terminal regulatory tyrosine of HsSrc with a high degree of specificity in vitro and in the fission yeast *Schizosaccharomyces pombe* (33, 34). As expected, we observed relatively specific phosphorylation of HsSrc by HsCsk in *S. cerevisiae.* However, CICsk extensively phosphorylated yeast proteins, suggesting that CICsk is much less specific than HsCsk (supplemental Fig. S7).

Patterns of yeast protein phosphorylation in the presence of Src were considerably more difficult to interpret (supplemental Fig. S6, A and B). Here, tyrosine phosphorylation levels did not always reflect yeast growth. For example, expression of highly active Src-tail mutants led to low yeast protein phosphorylation and slow growth, whereas expression of wild-type Src led to high yeast protein phosphorylation. Furthermore, co-expression of Src and Csk led to high phosphorylation and normal growth. Thus, although yeast growth is a useful and relatively straightforward readout of Src activity (30, 35), the relationship between activity, yeast protein phosphorylation, and growth is complex.

**Negative regulation of Src by Csk is present across holozoan organisms**

Our discovery of active Csk and Src proteins from *C. limacisporum* with an apparently intact negative regulatory mechanism suggested that this mechanism was present in ancestral holozoan Src and Csk. The reported lack of negative regulation of Src by Csk in choanoflagellates and filastereans could be explained by its loss in their common ancestor (Fig. 1) (21–24). The apparent lack of activity of Csk in filastereans, without signs of pseudogenization or non-functionalization, is harder to rationalize. The two filasterean species in which Csk has been characterized, *M. vibrans* and *C. owczarzaki,* diverged from metazoans ~900 million years ago (36, 37). Based on the estimated pseudogenization rate in unicellular organisms, preservation of the non-functional Csk coding sequence is highly unlikely (38).

Thus, we questioned whether the Csk-Src negative regulatory mechanism was really lost in Choanoflagellatea and Filasterea. To address this question, we analyzed the activity of Csk and Src proteins from *M. brevicollis* and *C. owczarzaki* using a luminescence-based in vitro kinase assay. *M. brevicollis* has four Src homologs, MbSrc1–4 (39). MbSrc1 and MbSrc4 have been characterized previously, revealing that MbSrc1 closely resembles HsSrc, whereas MbSrc4 appears to have an *M. brevicollis*–specific function (22, 40). *C. owczarzaki* has two Src orthologs, CoSrc1 and CoSrc2, with the latter having much higher activity (23). Thus, we selected MbSrc1 and CoSrc2 for further study. The activity of MbSrc1 and CoSrc2 toward the universal tyrosine kinase substrate poly(Glu4Tyr) was comparable with HsSrc (Fig. 4, A and B). We only obtained ~10 ng of protein per 1 ml of cell culture of CoSrc2, which prevented us from evaluating activity at high concentrations (supplemental Fig. S3D). Despite this limitation, we concluded that MbSrc1 and CoSrc2 were active, consistent with the results of the previous studies (22, 23). Next, we assessed the in vitro activity of MbCsk and CoCsk and found that both proteins were about as active as HsCsk (Fig. 4, C and D). Previously, MbCsk activity measured using the poly(Glu4Tyr) substrate was reported to be ~5-fold lower than HsCsk (22). CoCsk was reported to be inactive (23).

To determine whether MbCsk and CoCsk might inhibit Src activity, we quantified their ability to phosphorylate Src in vitro. We found that MbCsk phosphorylated a kinase-dead mutant of MbSrc1 (MbSrc1-KD, supplemental Table S1) with a level of activity comparable with that of HsCsk (Fig. 5A). MbCsk was unable to phosphorylate the kinase-dead, C-terminal regulatory tyrosine double mutant of MbSrc1 (MbSrc1-KD-tail), suggesting that MbCsk phosphorylation was specific. We were not able to obtain enough CoSrc2-KD or CoSrc2-KD-tail to use as CoCsk substrates. Instead, we measured the ability of CoCsk to phosphorylate HsSrc-KD and HsSrc-KD-tail. We found that CoCsk had appreciable activity when using HsSrc as a substrate, although the level of activity was lower than for HsCsk (Fig. 5B). Furthermore, CoCsk was unable to phosphorylate HsSrc-KD-tail, suggesting that CoCsk is also specific. The lower activity we observed for CoCsk might be due to our method of protein expression or the fact that HsSrc is not its native substrate.

Finally, we assessed the ability of Csk from *M. brevicollis* and *C. owczarzaki* to inhibit Src activity in our yeast assay. To our surprise, we found that both MbCsk and CoCsk appeared to inhibit Src activity (Fig. 5, C and D). Although proteins from *M. brevicollis* yielded unambiguous, human-like negative regulation, the results from *C. owczarzaki* proteins were somewhat different. For example, CoSrc2 caused a less severe growth
defect than the other Src proteins we analyzed. Interestingly, the growth defect caused by CoSrc2 was more severe when the C-terminal tyrosine was mutated to phenylalanine (CoSrc2-tail). This observation is consistent with the fact that the orthologous mutation is activating in HsSrc (41, 42). We note that neither HsSrc-tail nor MbSrc1-tail decreased growth compared with HsSrc or MbSrc1 in our yeast assay. However, HsSrc and MbSrc1 produced severe growth defects, and we speculate that our assay could not quantify additional decreases in these already low levels of growth. Finally, based on growth at 48 h, CoCsk-KD appeared to rescued growth (Fig. 5, C and D). However, examination of the entire growth curve led us to conclude that the apparent negative regulation in the absence of CoCsk kinase activity is not as strong as for ClCsk and most likely arises because of the moderate growth defect caused by CoSrc2 (supplemental Fig. S4).

We quantified protein expression and tyrosine phosphorylation in yeast by Western blotting (supplemental Fig. S6, C and D). Much like for human and C. limacisporum Src, we found that the expression of MbSrc1 and CoSrc2 mutants varied in an activity-dependent manner. MbCsk and CoCsk mutants were highly expressed. Like for HsCsk, but in contrast to ClCsk, MbCsk and CoCsk appeared to phosphorylate Src in a specific manner (supplemental Fig. S7). We attribute the HsCsk-like specificity of MbCsk and CoCsk to the fact that they all share a common domain architecture and key sequence features (Fig. 2).

Discussion

We characterized Src and Csk from C. limacisporum using both in vitro and yeast-based assays. We found that ClSrc and ClCsk are active tyrosine kinases. Furthermore, ClCsk phosphorylates ClSrc on its C-terminal regulatory tyrosine, and expression of ClCsk led to inactivation of ClSrc in yeast. Thus, the Csk-Src negative regulatory mechanism present in metazoans appears to be functional in proteins from C. limacisporum, a distantly related holozoan organism. These results prompted us to investigate whether the negative regulatory mechanism was present in Csk and Src proteins from the choanoflagellate M. brevicollis and the filasterian C. owczarzaki. In contrast to previous studies, we found that the negative regulatory mechanism appears to be functional in proteins from these species as well. Our findings suggest that negative regulation of Src by Csk is likely of ancient origin and could be common to all holozoans.

Although the sequence and domain architecture of ClSrc are similar to HsSrc, ClCsk differs from HsCsk in several important ways. Most prominently, ClCsk lacks an SH3 domain. In HsCsk, the kinase domain is intrinsically inactive, and contacts with the SH2 domain, SH3–SH2 linker, and SH2-kinase domain linker are required for activity (43, 44). In particular, the interaction of Arg-68 in the SH3–SH2 linker with Trp-188 in the kinase domain and Asn-148 and Met-150 with the N-lobe of the kinase domain are important for HsCsk activity (44). However, the residues that make these important interdomain contacts in HsCsk are not well conserved among other holozoan Csks, suggesting that stabilization of the kinase domain in the active conformation varies among Csk orthologs.

The activation loop is another region in which ClCsk differs from HsCsk. HsCsk is unusual among tyrosine kinases because its activation loop is several residues shorter than the activation loops of most tyrosine kinases. The short activation loop of HsCsk is thought to help explain its extraordinary specificity for HsSrc. Structural analyses suggested that the short activation loop of HsCsk disrupts the interactions necessary for peptide substrate recognition, and increasing the length of the loop by

Figure 4. M. brevicollis and C. owczarzaki Src and Csk are active tyrosine kinases. A–D, we measured the kinase activity of purified M. brevicollis (MbSrc1 and MbCsk) and C. owczarzaki (CoSrc2 and CoCsk) kinases using a luminescence assay. Tyrosine phosphorylation activity of MbSrc1 (A), CoSrc2 (B), MbCsk (C), and CoCsk (D) using poly(Glu4Tyr) as a substrate is shown. Human kinases (HsSrc and HsCsk) were included in each case as controls. Points represent the mean of three replicate luminescence assays, and error bars represent the standard deviation. KD, kinase-dead mutant (supplemental Table S1).
Figure 5. Csk from *M. brevicollis* and *C. owczarzaki* inhibit Src. We measured the kinase activity of purified *M. brevicollis* (MbCsk) and *C. owczarzaki* (CoCsk) kinases using a luminescence assay. A and B, tyrosine phosphorylation activity levels of MbCsk (A) and CoCsk (B) using their respective kinase-dead Src proteins as substrates. Mutants of the Src C-terminal regulatory tyrosine and human kinases (HsSrc and HsCsk) were included in each case as controls. Points represent the mean of three replicate luminescence assays, and error bars represent the standard deviation. C and D, optical density of yeast cultures 48 h after induction of kinase expression is shown for *M. brevicollis* (C) and *C. owczarzaki* (D). Under the no Src and no Csk conditions, yeasts were transformed with empty p416GAL1 or p415GAL1 vectors. Each point represents a replicate growth assay started from a distinct transformant, black bars represent the mean of the replicates, and error bars represent the standard deviation. KD, kinase-dead mutant; tail, C-terminal regulatory tyrosine mutant (supplemental Table S1).
two or more residues led to a 3-fold increase in activity toward peptide substrates (27). The CiCsk activation loop is six residues longer than the HsCsk loop and three residues longer than the HsSrc loop. The longer, HsSrc-like activation loop of CiCsk could explain why CiCsk phosphorylates a variety of proteins in yeast, whereas other Csks primarily phosphorylate Src. Our data also provide further evidence for the hypothesized relationship between activation loop length and substrate specificity and highlight the uniqueness of CiCsk. Thus, based on its sequence features and properties, we suggest that CiCsk might represent an evolutionary intermediate that has not yet fully developed the high substrate specificity seen in other Csk proteins.

In contrast, *M. brevicollis* and *C. owczarzaki* Csk have short, HsCsk-like activation loops and also displayed high substrate specificity toward Src in our yeast assay. Interestingly, *C. owczarzaki* has metazoan features, such as a full repertoire of integrin adhesion complex components (45) and temporal cell type differentiation (46) accompanied by changes in protein expression and phosphorylation (47). These metazoan-like features presumably require stringent regulation of the *C. owczarzaki* phosphotyrosine signaling network. This stringency could be reflected by the high specificity of CoCsk. We suggest that the low specificity of CiCsk might be indicative of a less elaborate phosphotyrosine signaling network and, consequently, a lower level of cellular complexity. However, further investigation of the cellular biology and life cycle of *C. limacisporum* is needed to understand the relationship between CiCsk specificity, phosphotyrosine network complexity, and cellular properties.

Unlike previous studies, we found that Csk from *M. brevicollis* and *C. owczarzaki* are about as active as human Csk *in vitro* (22, 23). In both our study and the previous studies, proteins were purified from *E. coli* and assayed using the universal poly(Glu4Tyr) substrate. However, in the other studies, MbCsk and CoCsk were expressed with a GST tag, whereas we tagged all of our proteins with the much smaller His tag. We speculate that the GST tag, which was not removed in the previous studies, might have compromised Csk activity. We note that HsCsk has routinely been expressed with a GST tag, but, in these cases, the GST tag was cleaved before biochemical analysis (27, 29).

In both previous studies, negative regulation of Src by Csk was primarily assayed in mammalian cell systems (22, 23). In both cases, Csk was not able to inhibit Src activity. In contrast, we used the budding yeast *S. cerevisiae* and observed negative regulation of Src by both MbCsk and CoCsk. To explain this disparity, we speculate that tyrosine kinases from distantly related organisms like *M. brevicollis* or *C. owczarzaki* might be subject to aberrant regulation by components of the innate phosphotyrosine signaling network of mammalian cells. By contrast, yeasts do not have tyrosine kinases or any tyrosine phosphorylation (47). These metazoan-like features might represent an evolutionary intermediate that has not yet fully developed the high substrate specificity seen in other Csk proteins.

Based on our findings, we suggest that negative regulation of Src by Csk is of ancient origin. Furthermore, this important negative regulatory mechanism appears to be conserved in proteins from a variety of holozoan organisms. To gain further insight into the evolution of this mechanism, we will need to study Csk and Src proteins from additional early holozoan organisms. Several examples are already available: Src and Csk orthologs have been found in the ichthyosporeans *Abeoforma whisleri*, *Anoebidium parasiticum*, and *Pirum gemmata* (12). As additional holozoan genomes are sequenced, this list will grow. In the future, Src and Csk should also be assessed in the native organismal context. The recent application of phosphoproteomics in *C. owczarzaki* (47) and DNA transformation and gene silencing in the ichthyosporean *Creolimax fragrantissima* (48) highlight how this could be done.

**Experimental procedures**

**Sequence annotation and alignments**

Representative kinases, including sequences from Src and Csk families, were collected from the Uniprot (49) and KinBase (http://www.kinase.com/kinbase/) databases. Orthologs were further identified using the OMA Browser (50). The final set consisted of 25 Src/Yes and 21 Csk/Chk sequences from 16 holozoan species: *Homo sapiens*, *Mus musculus*, *Monodelphis domestica*, *Gallus gallus*, *Xenopus tropicalis*, *Tetraodon nigroviridis*, *Danio rerio*, *Ciona intestinalis*, *Hydra vulgaris*, *Amphimedon queenslandica*, *Ephydatia fluviatilis*, *M. brevicollis*, *M. ovata*; *C. owczarzaki*, *M. vibrans*, and *C. limacisporum*. The domain architecture of these sequences was annotated using hidden Markov models compiled by Pfam, version 31.0 (51), and the software HMMER (52). Sequences were aligned using PROMALS3D (53). The alignments were guided using Src and Csk structures (PDB codes 2SRC and 1K9A, respectively). Src and Csk sequences experimentally analyzed here and elsewhere (12, 20–24) were used to construct separate alignments, shown in Fig. 2 and supplemental Fig. S1. Alignments were edited with Jalview (54).

**Phylogenetic analysis**

The full alignment, composed of 46 Src/Yes and Csk/Chk sequences, was processed using Gblocks (55), filtering for conserved positions with at least 23 sequences, a maximum of 12 non-conserved contiguous sites, and a minimum length of five sites per block. These parameters resulted in 324 sites and 14 blocks. We selected the fittest evolutionary model using the software Prttest version 3.0 (56). A phylogenetic tree was constructed using maximum likelihood, the LG substitution matrix, and G+I model parameters, as implemented in RAxML version 8.2.8 (57). Bootstrap support values were calculated over 100 iterations.

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Src regulation by Csk

Cloning and mutagenesis

Csk and Src genes were purchased as gBlocks gene fragments from Integrated DNA Technologies (Coralville, IA; supplemental Table S2). The genes were codon-optimized for the yeast S. cerevisiae using the codon optimization tool available on the Integrated DNA Technologies website. Genes were amplified in a PCR reaction containing 1× Phusion HF buffer (New England Biolabs, Ipswich, MA), 0.2 mM dNTPs (Qiagen, Germantown, MD), 0.4 mM each forward and reverse primers, 10 ng of gBlocks gene fragment, and 1 Unit of Phusion HF DNA polymerase (New England Biolabs). The PCR reaction was incubated for 2 min at 98 °C, followed by 25 cycles of 10 s at 98 °C, 15 s at 60 °C, and 60 s at 72 °C and a final extension at 72 °C for 5 min. Primers were designed to add 20-base pair overlaps for Gibson assembly cloning into the appropriate expression vector (Ref. 58, New England Biolabs). For recombinant protein expression in E. coli, genes were cloned into the pET16b vector (EMD Millipore, Billerica, MA) digested with NdeI and XhoI (New England Biolabs), resulting in N-terminal fusion to a decahistidine tag. For protein expression in yeast, genes were amplified in a two-step PCR to add HA or Myc tags to the N termini of Csk and Src, respectively. Genes were cloned into either p415GAL1 (Csk) or p416GAL1 (Src) vectors digested with SpeI and HindIII (New England Biolabs) (59). Src genes were cloned without the amino acid N-terminal of the SH3 domain, corresponding to the first 86 residues in HsSrc (supplemental Fig. S1, arrow). Expression of full-length Src in E. coli causes difficulties, and it has traditionally been expressed in the truncated form (27, 28). The primers used to amplify DNA fragments are listed in supplemental Table S3.

All PCR products were analyzed on 0.7% Tris borate-EDTA-agarose gels (UltraPure agarose, Thermo Fisher Scientific, Waltham, MA) and purified by gel extraction using a QIAquick gel extraction kit (Qiagen). Purified DNA fragments and restriction-digested vectors were mixed in a 2:1 DNA:vector ratio with 1× Gibson Assembly Master Mix (New England Biolabs) and incubated at 50 °C for 1 h. Then the reaction products were transformed into chemically competent Top10F’ cells and plated on lysogeny broth (LB) plates containing 100 µg/ml ampicillin. After overnight incubation at 37 °C, colonies were selected from the LB plate and grown overnight in LB medium at 37 °C with shaking at 220 rpm. Plasmid DNA was extracted using a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). All DNA constructs were verified using Sanger sequencing (Thermo Fisher Scientific).

Mutations were introduced using the inverse PCR (60). Here, non-overlapping, adjacent primers were designed, with the mutation located at the 5’ end of the forward primer (supplemental Table S3). Inverse PCR reactions consisted of 500 pg of template, primer (2.5 µM each), and 1× Kapa HiFi Hot Start Ready Mix (Kapa Biosystems, Wilmington, MA). The PCR reaction was incubated for 3 min at 95 °C, followed by 20 cycles of 20 s at 98 °C, 15 s at 60 °C, and 60 s per kilobase at 72 °C and a final extension at 72 °C for 10 min. PCR products were purified by gel extraction as described above. Each purified PCR product was phosphorylated using T4 polynucleotide kinase in T4 ligase buffer (New England Biolabs) for 30 min at 37 °C. The kinase was heat-inactivated for 20 min at 65 °C. The phosphorylated product was subsequently ligated using T4 ligase (New England Biolabs) in T4 ligase buffer for between 3 h and 16 h at 16 °C. Ligation products were transformed, isolated, and verified as described above.

Protein expression

The pET16b vector constructs were transformed into chemically competent BL21(DE3) E. coli cells (Thermo Fisher Scientific). Transformed cells were plated onto LB plates containing 100 µg/ml ampicillin and incubated overnight at 37 °C. Single colonies were grown over night in 10 ml of terrific broth medium containing 100 µg/ml ampicillin at 37 °C with shaking at 220 rpm. From each overnight culture, 5 ml was used to inoculate 250 ml of terrific broth medium containing 100 µg/ml ampicillin. Cells were grown at 37 °C with shaking at 200 rpm to an A600 of 1.2. Cell cultures were cooled to 18 °C for 1 h. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.2 mM. Protein expression was carried out for 16 h at 18 °C and with shaking at 200 rpm. Cells were harvested by centrifugation at 4225 × g for 15 min. The supernatant was discarded, and pellets were stored at −20 °C. In cases where protein expression levels were low, vectors were transformed into chemically competent BL21(DE3) E. coli cells expressing the tyrosine phosphatase YopH and the chaperone protein GroEL (61). The transformed cells were plated onto LB plates containing 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, and 50 µg/ml spectinomycin. Protein expression was carried out as described above.

Protein purification

Cell pellets were dissolved in lysis buffer (50 mM HEPES (pH 8), 10 mM imidazole, 300 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF). Cells were lysed by sonication for 3 × 1 min with a 0.9-s pulse and a 37% amplitude (B. Braun Labsonic U/B, Braun Biotech International, Melsungen, Germany). The insoluble fraction was removed by centrifugation at 4 °C for 30 min at 10,000 × g. HisPur cobalt resin (Thermo Fisher Scientific) was washed with lysis buffer, added to the cleared cell lysate, and incubated for 90 min at 4 °C on a rotating wheel. The resin was collected by centrifugation at 4 °C for 2 min at 700 × g. The supernatant was carefully removed, and the resin was transferred to an Econo-Pac chromatography column (10 ml, BioRad). The resin was washed with 10 ml of lysis buffer and 10 ml of wash buffer (lysis buffer containing 20 mM imidazole). Protein was eluted with 3 ml of elution buffer (lysis buffer containing 150 mM imidazole). Eluate was dialyzed to dialysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 5% glycerol, and 1 mM DTT) using a Slide-A-Lyzer Dialysis Cassette G2 (20,000,20,000-Da molecular weight cut-off, 3-ml capacity; Thermo Fisher Scientific). Dialysis was carried out at 4 °C. The dialysis buffer was changed twice at 90-min intervals, and then dialysis was continued overnight. The dialysate was centrifuged at 4 °C for 5 min at 3000 × g to remove any precipitate. The protein was aliquoted and stored at −80 °C. The molecular size and purity of the protein were analyzed by SDS-PAGE and subsequent Coomassie Brilliant Blue staining (supplemental Fig. S3). A molecular marker (Spectra Multicolor Broad Range Protein Markers, Thermo Fisher Scientific) was included for size determination.
Ladder, Thermo Fisher Scientific) was used for size determination. The protein concentrations in the eluted fractions were determined using the theoretical molar absorption coefficient of the mutants at 280 nm and the estimated molecular weight as determined from the amino acid sequence using the ExpASy Prot Param tool (http://www.expasy.org/protparam). The A_280 values were measured using a NanoDrop 2000c (Thermo Fisher Scientific). In cases where protein impurity was high, SDS-PAGE band intensities were compared with band intensities of known protein amounts using the ImageJ gel analysis tool.

Proteins expressed in the presence of YopH required a second purification step by anion exchange to remove YopH. After elution from the cobalt resin, the protein was dialyzed into anion exchange buffer (50 mM Tris-HCl (pH 8), 20 mM NaCl, 5% glycerol, and 1 mM DTT). Dialysis was carried out as described above. The dialyzed protein was then loaded onto a Pierce Strong Anion Exchange Spin Column (Thermo Fisher Scientific). The protein was eluted with anion exchange buffer containing increasing amounts of NaCl (50–1000 mM in 50 mM steps). Protein-containing fractions were determined by SDS-PAGE and stored as described above.

**Kinase activity assay**

Kinase activity was measured using the Kinase-GLO Plus luminescent kinase assay (Promega, Madison, WI). Varying concentrations of kinase were incubated in 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 5 mM MnCl₂, 0.1 mM EDTA, 0.1 mM Na₃VO₄, 0.1% β-mercaptoethanol, and 1 μM ATP in a total volume of 25 μL. Kinase reactions were incubated with shaking for 30 min at 30 °C in a Synergy H1 multimode reader (Biotek, Winooski, VT), after which 25 μL of Kinase-GLO reagent was added. After another 10 min of incubation, luminescence was recorded. To test for tyrosine kinase activity of Csk and Src proteins individually, 100 ng/μL poly(Glu,Tyr) (Glu:Tyr (4:1)) (Sigma-Aldrich, St. Louis, MO) was included in the kinase reaction. To test for the ability of each Csk to phosphorylate its corresponding Src, 100 nM Csk and 0–5000 nM kinase-dead Src were used.

The mean and standard deviation of three independent measurements were calculated. Luminescence values of samples containing kinase and substrate were subtracted from the luminescence value of sample containing no kinase and substrate. Luminescence values were converted into nanomolar phospho-transferred using an ATP standard curve.

**Yeast growth assay**

Using the lithium acetate method (62), S. cerevisiae strain BY4741 was double-transformed with different combinations of p415GAL1 and p416GAL1 plasmids carrying mutants of Csk and Src, respectively. Transformed cells were plated onto c-LEU-URA plates and incubated for 2 days at 30 °C. Single colonies were inoculated in 100 μL of c-LEU-URA media containing 2% glucose. Cells were grown in a Falcon 96-well flat-bottom non-tissue culture–treated plate (Corning, Corning, NY) for 20 h at 30 °C with shaking at 220 rpm. Cells were collected by centrifugation at 3000 × g for 5 min and washed twice with deionized water. Half of the cells were used to inoculate 100 μL of c-LEU-URA media containing 2% raffinose. Cells were incubated for 2 h at 30 °C with shaking at 220 rpm. Cells were then used to inoculate 100 μL of c-LEU-URA media containing 2% galactose with a starting A₆₀₀ of 0.05. This culture was incubated for 48 h at 30 °C in a Synergy H1 multimode reader (Biotek) with orbital shaking. Cell density was measured every 30 min by measuring absorbance at 600 nm. Data from six biological replicates were collected. Replicates that did not show any growth or appeared to be contaminated were excluded from the analysis.

**Western blotting**

Single colonies were inoculated into 5 ml of c-LEU-URA media containing 2% glucose. Cells were grown for 2 h at 30 °C on a rotating wheel. Cells were then collected by centrifugation at 4000 × g for 5 min and washed twice with deionized water. Half of the cells were used to inoculate 5 ml of c-LEU-URA media containing 2% raffinose. Cells were incubated for 2 h at 30 °C on a rotating wheel. Cells were used to inoculate 5–50 ml of c-LEU-URA media containing 2% galactose with a starting A₆₀₀ of 0.05. Cells were incubated for 22 h at 30 °C with shaking at 220 rpm. 5–10 Optical density units of cells were collected by centrifugation at 4000 × g for 5 min. Cell pellets were stored at −80 °C. Each cell pellet was resuspended in 200 μL of 10 mM MOPS (pH 6.8), 10 mM EDTA, 8 M urea, 1% SDS, 0.01% bromophenol blue, protease inhibitor mixture (Sigma-Aldrich), and 5% β-mercaptoethanol. Cells were lysed by vortexing for 3 min in the presence of 100 μL of acid-washed glass beads (0.5 mm). Lysed cells were centrifuged at 4 °C for 5 min at 20,000 × g. The supernatant was transferred to a fresh tube and incubated for 10 min at 65 °C with shaking at 2000 rpm. The total protein concentration was determined using a Qubit protein assay kit and Qubit 2.0 fluorometer (Thermo Fisher Scientific).

Cell lysate containing 10 μg of protein was separated on a NuPAGE 4–12% Bis-Tris polyacrylamide gel (Thermo Fisher Scientific). Protein was transferred to a BioTrace NT nitrocellulose transfer membrane (Pall Life Sciences, Port Washington, NY). The nitrocellulose membrane was washed in TBS containing 0.1% Tween (TBST) for 5 min at room temperature. The membrane was blocked with either 5% milk or 5% BSA in TBST for 1 h at room temperature. The membrane was washed five times for 5 min in TBST and then incubated in primary antibody overnight at 4 °C. The membranes were washed five times for 5 min at room temperature in TBST and then incubated in secondary antibody, where necessary, for 1 h at room temperature. The membrane was washed again five times in TBST and then incubated in 1 ml of SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific) for 5 min. Finally, the membrane was exposed to film. We used the following antibodies: anti-c-Myc antibody (9E10, Abcam, Cambridge, MA) at 1:1000 in 5% milk in TBST in combination with 1:10,000 diluted Amersham Biosciences ECL mouse IgG and HRP-linked whole antibody from sheep (GE Healthcare Life Sciences, Marlborough, MA) to detect Myc-tagged Src proteins, anti-HA-peroxidase (Sigma-Aldrich) at 1:50,000 in 5% milk in TBST to detect HA-tagged Csk proteins, anti-phosphotyrosine mouse monoclonal antibody (Tyr(P)-100, Cell Signaling Technologies, Danvers, MA) used at 1:2000 in 5% BSA in TBST in combination with 1:10,000 diluted Amersham Biosciences ECL mouse IgG.
and HRP-linked whole antibody from sheep to detect total tyrosine phosphorylation, and anti-GAPDH antibody (HRP, Abcam) at 1:1000 in 5% milk in TBST as a loading control.

**Author contributions**—B. T. and D. M. F. conceived the study and designed the experiments. E. F. conducted the phylogenetic analysis. B. T. performed the experiments. B. T., E. F., and D. M. F. wrote and edited the manuscript.

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