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Regulation of Src and Csk Nonreceptor Tyrosine Kinases in the Filasterean *Ministeria vibrans*

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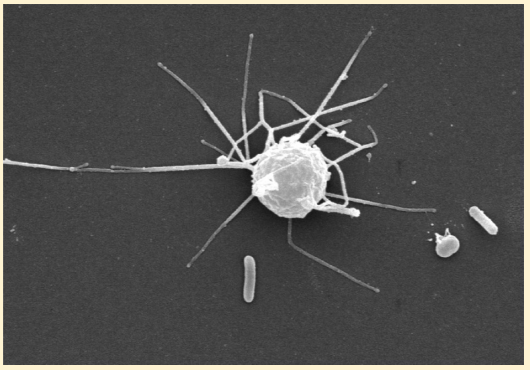
Supporting Information

ABSTRACT: The development of the phosphotyrosine-based signaling system predated the evolution of multicellular animals. Single-celled choanoflagellates, the closest living relatives to metazoans, possess numerous tyrosine kinases, including Src family nonreceptor tyrosine kinases. Choanoflagellates also have Csk (C-terminal Src kinase), the enzyme that regulates Src in metazoans; however, choanoflagellate Csk kinases fail to repress the cognate Src. Here, we have cloned and characterized Src and Csk kinases from *Ministeria vibrans*, a filasterean (the sister group to metazoans and choanoflagellates). The two Src kinases (MvSrc1 and MvSrc2) are enzymatically active Src kinases, although they have low activity toward mammalian cellular proteins. Unexpectedly, MvSrc2 has significant Ser/Thr kinase activity. The Csk homologue (MvCsk) is enzymatically inactive and fails to repress MvSrc activity. We suggest that the low activity of MvCsk is due to sequences in the SH2−kinase interface, and we show that a point mutation in this region partially restores MvCsk activity. The inactivity of filasterean Csk kinases is consistent with a model in which the stringent regulation of Src family kinases arose more recently in evolution, after the split between choanoflagellates and multicellular animals.



Multicellular animals evolved from unicellular ancestors more than 600 million years ago.[1] This critical transition required the presence of systems for cellular adhesion, cell−cell communication, and intracellular signal transduction. The cellular components that mediate these processes have surprisingly deep phylogenetic roots; much of the machinery is present in three distinct unicellular lineages (choanoflagellates, filastereans, and ichthyosporeans) that are closely related to metazoans.[2−5] Thus, many of the genomic innovations that were required for multicellularity were already present in the immediate ancestors of metazoans.

In animal cells, tyrosine phosphorylation controls vital processes such as cell growth, differentiation, and survival.[6,7] The components of phosphotyrosine (pTyr)-based signal transduction (receptor and nonreceptor tyrosine kinases, pTyr-binding domains, and tyrosine phosphatases) are abundant in choanoflagellates, the closest living relatives to metazoans. The genome of the choanoflagellate *Monosiga brevicollis* encodes a diverse set of tyrosine kinases (TKs), including homologues of the mammalian Src, Csk, Abl, and Tec nonreceptor kinases.[8−11] The *M. brevicollis* Src family kinase MbSrc1 has enzymatic properties similar to those of its mammalian counterpart and can functionally replace c-Src in mammalian cells.[8,12] On the other hand, the regulatory properties of the two Src kinases are different. Whereas mammalian c-Src is inhibited by phosphorylation on a C-terminal tyrosine (Y527) by Csk, phosphorylation of MbSrc1 at the equivalent site by the *M. brevicollis* Csk does not significantly inhibit activity.[12] Similar results were observed in the choanoflagellate *Monosiga ovata*.[13] This lack of Csk-mediated Src regulation is more pronounced in the filasterean *Capsaspora owczarzaki*, which represents a sister group to metazoans and choanoflagellates.[14−16] In *Capsaspora*, the Csk homologue (CoCsk) does not have any detectable tyrosine kinase activity and is incapable of phosphorylating or inhibiting the two *Capsaspora* Src kinases (CoSrc1 and CoSrc2).[17]

The previous results for *M. brevicollis* and *C. owczarzaki* suggest that, while Src and Csk TKs evolved prior to the divergence of filastereans from choanoflagellates and metazoans, the function of Csk as a negative regulator of Src evolved afterward.[18] To understand the early evolution of the Src−Csk signaling pair, it is necessary to analyze kinases from additional organisms. Apart from *Capsaspora*, the only other known filasterean species is *Ministeria vibrans*, a free-living marine protist (Figure 1A).[14] A polymerase chain reaction (PCR)-based survey in *Mi. vibrans* identified 15 genes encoding tyrosine kinases (seven receptor TKs and eight nonreceptor TKs).[5] The nonreceptor tyrosine kinases were further subdivided into six families, most of them representing groups common to both *Capsaspora* and mammalians (Src, Csk, Abl,

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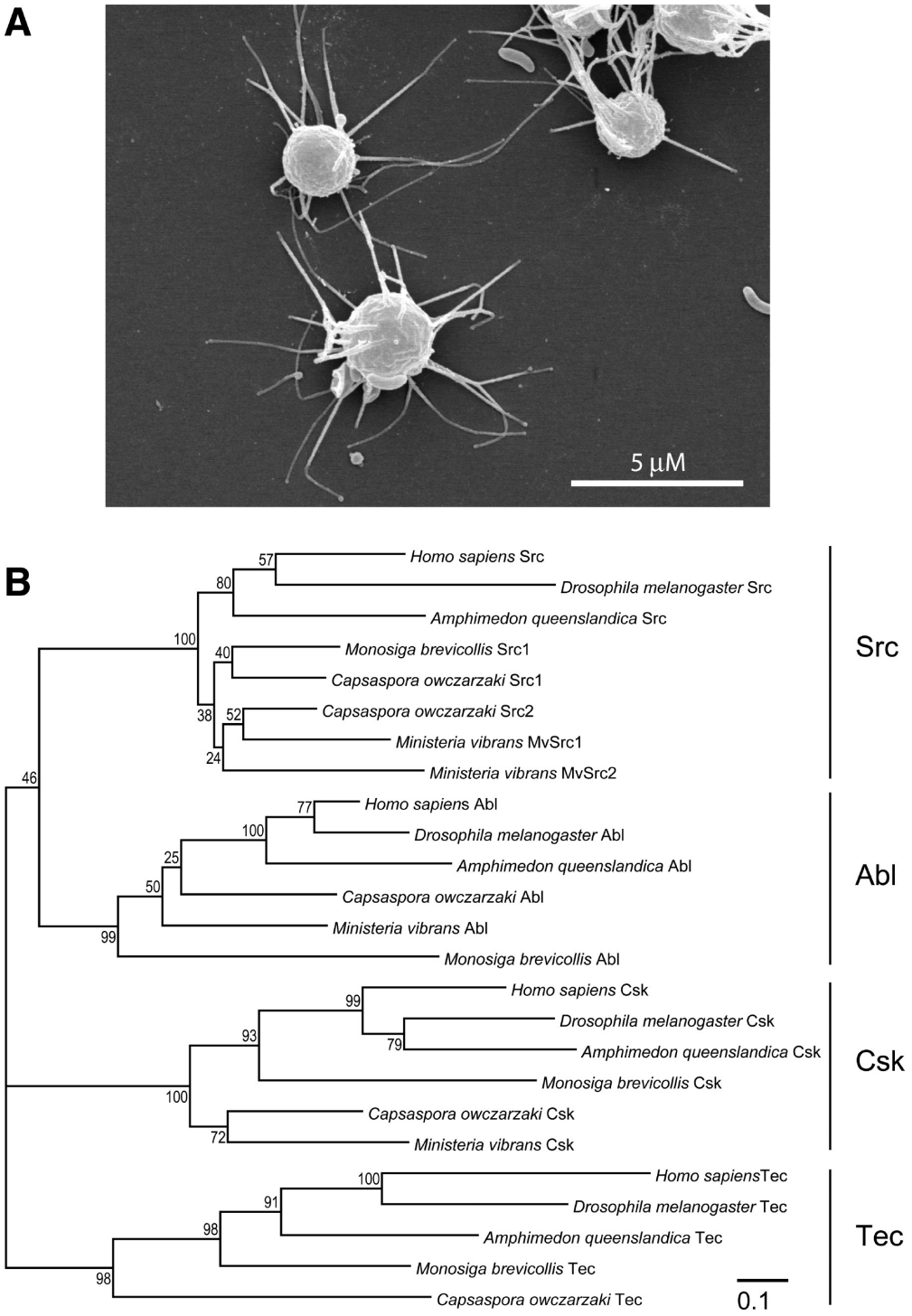

Figure 1. (A) *Mi. vibrans.* (B) Phylogenetic tree comprising four cytoplasmic TK families. An alignment of 363 amino acid sites, which covers SH3, SH2, and TK domains, was used for the tree inference. The Bootstrap values with 200 replicates are shown as branches. The scale bar shows 0.1 substitution per site.

Fak, and Fes), with one kinase (CTK1) that consists of a tyrosine kinase region without any other modular domains. The eight receptor TKs in *Mi. vibrans* form a unique family designated RTK1, which does not share domain architecture with any other known RTK family, including those of *Capsaspora*.[5]

In this study, we have characterized the two Src family kinases from *Mi. vibrans* (designated MvSrc1 and MvSrc2) and the single Csk homologue (MvCsk). We have cloned, expressed, and purified the proteins to compare them to their mammalian counterparts and to those in *Capsaspora* and *M. brevicollis*. MvSrc1 and McSrc2 have a domain arrangement similar to that of mammalian and *Capsaspora* Src kinases, and the regulatory tyrosine residues are conserved. MvSrc1 and MvSrc2 are enzymatically active toward tyrosine-containing peptides and proteins, and MvSrc2 unexpectedly also has significant serine/threonine kinase activity. As seen previously for the *Capsaspora* homologue of Csk, MvCsk displays no

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detectable activity toward MvSrc1, MvSrc2, or a general tyrosine kinase substrate. Mutation of a single residue in the SH2−kinase interface of MvCsk restores partial activity. Thus, while choanoflagellate Csk enzymes are active (but unable to regulate Src kinases), the known filasterean Csk enzymes appear to be inactive. The tight regulation of Src family kinases by Csk appears to have arisen more recently in evolution, after the split between choanoflagellates and multicellular animals.

MATERIALS AND METHODS

Reagents and Antibodies. Nickel-nitriloacetic acid resin was purchased from Qiagen. The anti-phosphotyrosine antibody (clone 4G10) was purchased from Millipore. The anti-FLAG antibody, Anti-FLAG M2 Affinity Gel, leupeptin, aprotinin, phenylmethanesulfonyl fluoride, sodium vanadate, and bovine serum albumin (BSA) were from Sigma. The anti-V5 antibody and glutathione-agarose-linked beads were purchased from Invitrogen. The QuikChange site-directed mutagenesis kit was purchased from Agilent.

Cell Culture. A living culture of *Mi. vibrans* was purchased from the American Type Culture Collection (ATCC) and maintained at 17 °C in ATCC medium 1525.

cDNA Cloning and Alignments. *Mi. vibrans* total RNA was extracted by Trizol (Lifetech), and cDNA was generated by SuperScript III reverse transcriptase (Lifetech). The MvSrc1, MvSrc2, and MvCsk cDNAs were amplified by polymerase chain reaction with degenerate primers, and the full-length cDNAs were obtained by rapid amplification of cDNA ends (RACE).[5] Amino acid sequences were aligned with ClustalW and formatted by BOX-SHADE (version 3.3.1 by K. Hofmann and M. D. Baron) in UCSK workbench 3.2. Phylogenetic tree analysis was performed by the Maximum Likelihood (ML) method. RAxML[19] was used with the LG model. The amino acid substitution rate was shaped by the Γ distribution implemented by the program.

For insect cell expression, MvSrc1 and MvSrc2 were cloned into the BamHI and XbaI sites of pFastbac-HtC (Invitrogen), inserting residues 95−555 and 79−596, respectively, to produce N-terminally His-tagged enzymes. For mammalian expression, full-length MvSrc1 and MvSrc2 (555 and 596 residues, respectively) were subcloned into the XbaI and BamHI restriction sites of p3X-FLAG-CMV (Sigma) to produce N-terminally FLAG-tagged enzymes. The cDNA for MvCsk (456 residues) was subcloned into pGEX-4T-1 (GE Healthcare) using EcoRI and XhoI restriction sites for bacterial expression. A point mutation was made in the pGEX-MvCsk construct by site-directed mutagenesis (G236N). V5-tagged MvCsk was expressed in mammalian cells by being subcloned into the BamHI and XbaI sites of pEF1/V5-HisA (Invitrogen).

Protein Expression and Purification. His-tagged MvSrc1 and MvSrc2 proteins were expressed in *Spodoptera frugiperda* (Sf9) insect cells using the Bac-to-Bac system (Invitrogen). Recombinant MvSrc1 and MvSrc2 baculoviruses were used to infect 600 mL of Sf9 insect cells in spinner flasks for 72 h. Cells were lysed in a French pressure cell, and His-tagged proteins were purified on 4 mL of nickel-nitrilotriacetic acid resin, as described previously.[17] The proteins were stored at −20 °C in 40% glycerol. Full-length GST-tagged MvCsk was expressed in 1 L of *Escherichia coli* BL21(DE3) cell cultures. Cells were lysed in a French pressure cell, and MvCsk was purified on glutathione-agarose-linked beads.[17] GST-MvCsk was eluted with 20 mM glutathione in 50 mM Tris (pH 8.0) and stored in 40% glycerol at −20 °C.

Mammalian Cell Transfection and Western Blotting. Mammalian HEK293T and Src/Yes/Fyn deficient cells (SYF) were maintained in Dulbecco's modified Eagle's medium (Cellgro Mediatech, Inc.) with 10% fetal bovine serum (Sigma) at 37 °C in 5% CO_2. Cells were transfected with TransIT transfection reagent at 50% confluency with a ratio of 1:3 (DNA:TransIT). The cells were harvested 48 h later and lysed in buffer containing 25 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% NP40, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 mM Na_3VO_4 for 1 h at 4 °C. Lysates were centrifuged at 14000*g* for 15 min at 4 °C, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), and transferred onto a polyvinylidene difluoride membrane. Proteins were detected by Western blotting with anti-phosphotyrosine, anti-FLAG, and anti-V5 antibodies.

Tyrosine Kinase Assays. The activity of MvSrc1 and MvSrc2 was assayed using [γ-^{32}P]ATP in phosphocellulose paper binding assays.[20] Reactions (25 μL) were conducted in 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 0.25 mM ATP, with varying concentrations of peptide substrates and 100−500 cpm/pmol of [γ-^{32}P]ATP. The following peptides were used: Src peptide, AEEEEIYGEFEAKKKKG; EGFR peptide, AEEEEYFELVAKKKG; Abl peptide, EAIYAAPFAKKKG;[21,22] and Kemptide, LRRASLG.[23] Substrate targeting was tested with the following peptides: SH2 substrate, RRLEDAIYAAGGGGGEPPQpYEEIG; SH2 control, RRLEDAIYAAGGGGGEPPQFEEIG; SH3 substrate, AEEEIYGEFGGRGAAPPPPPVPRGRG; and SH3 control, AEEEIYGEFGGRGAAAAAAAVPRGRG.[24]

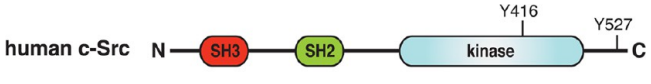
The activities of mammalian Csk, MbCsk, and MvCsk were measured using [γ-^{32}P]ATP and poly(Glu_4-Tyr) as a substrate.[25] After varying lengths of time, aliquots of the reaction mixtures were spotted on Whatman 3MM filter paper squares. The filters were washed with 5% trichloroacetic acid at 55 °C prior to liquid scintillation counting. Phosphorylation of MvSrc1 and MvSrc2 by MvCsk (and of c-Src by Csk) was tested by incubation with [γ-^{32}P]ATP for 30 min. The reactions were terminated by the addition of SDS−PAGE sample buffer. The samples were analyzed by 10% SDS−PAGE and autoradiography. To test for MvCsk-mediated regulation of MvSrc1 and MvSrc2, the enzymes were incubated together with ATP for 15 min at 30 °C. Aliquots from this mixture were then tested for Src activity with [γ-^{32}P]ATP and Src peptide. This reaction proceeded for 5 min at 30 °C, and Src activity was quantified by the phosphocellulose paper binding assay as described above.

Immunoprecipitation/Kinase Assays. Cell lysates (2 mg of total protein) were incubated with 4 μg of the HRP-anti-FLAG antibody (Sigma) for 1 h at 4 °C. The complexes were captured by a 2 h incubation at 4 °C with a 30 μL bed volume of protein A agarose (Roche). After being extensively washed with PBS (Mediatech) containing 1 mM sodium orthovanadate, each agarose−protein complex was divided into three tubes. Duplicates were studied in a radioactive kinase assay using Src peptide (AEEEIYGEFEAKKKKG). Activities of the proteins were calculated and expressed as the picomoles of phosphate transferred. The remaining samples were analyzed by SDS gel electrophoresis, transferred to a PVDF membrane (Millipore), and probed with the HRP-anti-FLAG antibody to confirm equal immunoprecipitation.

RESULTS

Cloning MvSrc1 and MvSrc2 from *Mi. vibrans*. We used PCR to isolate cDNAs encoding two Src-like kinases from *Mi.*

vibrans. These kinases, which we have designated MvSrc1 and MvSrc2, are closely related to the Src family kinases from the filasterean *C. owczarzaki* and the choanoflagellate *M. brevicollis*, as shown in the phylogenetic tree in Figure 1B. The *Mi. vibrans* Src kinases have the same basic domain structure as *M. brevicollis* MbSrc1, *Capsaspora* CoSrc1 and CoSrc2, and mammalian Src: all contain an N-terminal myristoylation sequence, followed by a unique region, SH3, SH2, and catalytic kinase domains (Figure 2 and Figure S1 of the Supporting Information). This domain arrangement is well-conserved, because in addition to functioning in substrate recognition and targeting, the SH3 and SH2 domains are involved in autoinhibitory interactions.[26−28] The kinase domains of MvSrc1 and MvSrc2 are approximately 60% identical in amino acid sequence with the human c-Src kinase domain, while the noncatalytic domains have lower levels of identity (SH3, 55.2 and 44.1% for MvSrc1 and MvSrc2, respectively; SH2, 37.6 and 44.6% for MvSrc1 and MvSrc2, respectively) (Figure 2). In mammalian c-Src, phosphorylation of a tyrosine in the C-terminal tail (Y527; chicken c-Src numbering) by Csk produces an intramolecular interaction with the SH2 domain, leading to enzyme inhibition.[7,26,29,30] This C-terminal tyrosine is conserved in MvSrc1 and MvSrc2 (Y546 and Y589, respectively). Activation of the Src family kinases occurs through autophosphorylation of a tyrosine in the activation loop (Y416; chicken c-Src numbering). This is facilitated by disruption of the autoinhibitory interactions through either binding of a ligand to the SH2 or SH3 domains or dephosphorylation of the C-terminal tyrosine.[26,27,31] Autophosphorylation also regulates the Src kinases from *Capsaspora* and *M. brevicollis*.[12,17] MvSrc1 and MvSrc2 both contain a tyrosine in the activation loop (Y435 and Y478, respectively), corresponding to the c-Src autophosphorylation site.



Percent amino acid identity			
MvSrc1	55.2	37.6	63.6
MvSrc2	44.1	44.6	59.7
MbSrc1	62.1	47.3	61.1
CoSrc1	65.5	45.1	61.4
CoSrc2	58.6	40.7	63.3

Figure 2. Homology between human c-Src and unicellular SFKs. The Src domain structure is shown schematically. The percent amino acid identity among the SH3, SH2, and kinase domains of human c-Src and various unicellular Src family kinases are shown below the structure. The positions of the autophosphorylation site (Y416) and the negative regulatory site (Y527) are indicated.

MvSrc1 and MvSrc2 Are Active Kinases-. We expressed MvSrc1 and MvSrc2 in Sf9 insect cells using recombinant baculoviruses and purified the enzymes by nickel affinity chromatography. We tested for tyrosine kinase activity using a synthetic Src substrate peptide. Both enzymes were active, with MvSrc1 displaying approximately 10−20-fold higher activity than MvSrc2 over a range of enzyme concentrations (Figure 3A). The specific activity of MvSrc1 measured in these experiments (47.5 pmol min^{-1} μg^{-1}) is lower than the activity previously measured for autophosphorylated mammalian c-Src (430 pmol min^{-1} μg^{-1})[32] or *M. brevicollis* MbSrc1 (145 pmol min^{-1} μg^{-1}),[12] although it is comparable to the activity of the mammalian Src-like kinase Brk (37 pmol min^{-1} μg^{-1}).[22] The specific activity of MvSrc2 is substantially lower than the specific activities of these other kinases (2.4 pmol min^{-1} μg^{-1}). The activity of MvSrc1 was somewhat higher in Mn^{2+}-containing buffers than in Mg^{2+}-containing buffers, while MvSrc2 did not display any preference (Figure S2 of the Supporting Information). We investigated the substrate specificities of MvSrc1 and MvSrc2 using synthetic peptides containing substrate motifs for a variety of kinases (Figure 3B). The substrate preference for MvSrc1 was similar to the preferences previously observed for mammalian, *M. brevicollis*,

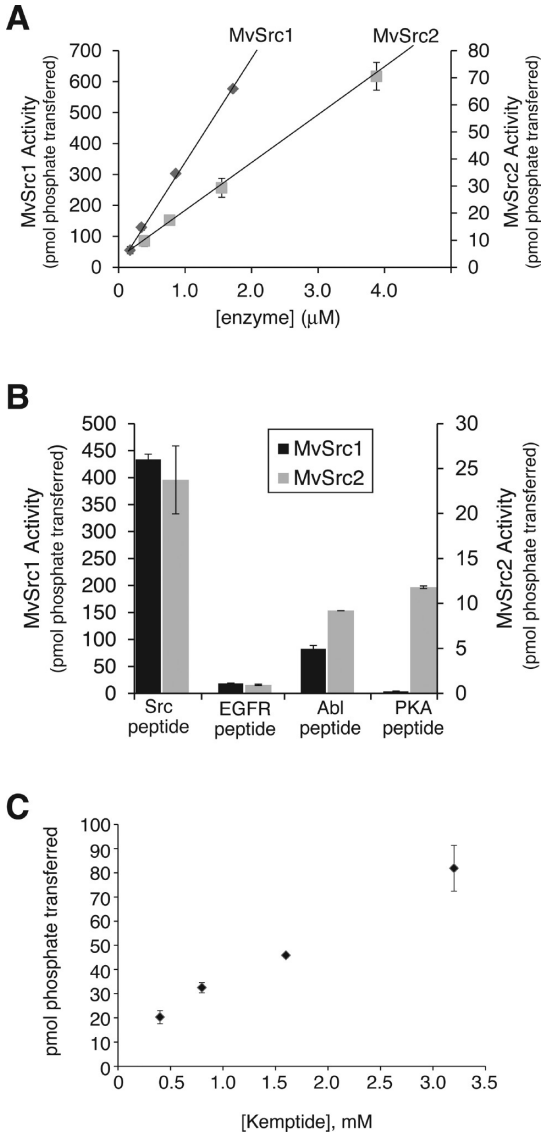


Figure 3. MvSrc1 and MvSrc2 are active tyrosine kinases. (A). The activities of MvSrc1 and MvSrc2 at varying enzyme concentrations were measured with 0.7 mM Src peptide and 0.25 mM [γ-^{32}P]ATP using the phosphocellulose paper binding assay. Reactions proceeded for 9 min at 30 °C. Note that two different activity scales were used for MvSrc1 and MvSrc2. (B). MvSrc1 and MvSrc2 substrate specificities were investigated using synthetic peptides incorporating recognition motifs from four protein kinases. The enzymes were incubated with 0.25 mM [γ-^{32}P]ATP and 0.8 mM peptide, and reactions proceeded for 8 min at 30 °C. (C) The activity of MvSrc2 was investigated at various concentrations of Kemptide, a PKA substrate. The concentration of enzyme was 2 μM, and the concentration of [γ-^{32}P]ATP was 0.25 mM. Reactions proceeded for 9 min at 30 °C.

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and *Capsaspora* Src kinases,[12,17] with the highest activity toward the Src peptide substrate. MvSrc2 preferred the Src substrate, but it also displayed a significant amount of activity toward Kemptide (LRRASLG), a substrate for protein kinase A, a serine/threonine kinase (Figure 3B). In contrast, MvSrc1 was inactive toward Kemptide. Phosphorylation of Kemptide by MvSrc2 was confirmed by matrix-assisted laser desorption ionization time of flight (data not shown). The activity of MvSrc2 toward Kemptide showed a dependence on peptide concentration (Figure 3C). We were unable to achieve saturation in these experiments, but the K_m value appears to be in the millimolar range. The level of phosphorylation of the Src substrate or Kemptide by MvSrc2 was reduced to background levels in the presence of 200 nM dasatinib, a small molecule Src inhibitor (data not shown). Serine/threonine kinase activity has not previously been reported for a purified mammalian Src kinase or for a Src kinase from any of the unicellular eukaryotes previously studied.

The SH2 and SH3 domains of mammalian Src family kinases play important roles in substrate recognition.[7,33] Thus, protein or peptide substrates containing SH2 or SH3 ligands (or both) are phosphorylated much more efficiently than substrates lacking the ligand sequences. MvSrc1 and MvSrc2 displayed higher activity toward a peptide with an SH3 ligand than a control peptide in which the key proline residues within the SH3-binding sequence were changed to alanines (Figure 4A,B). In contrast, MvSrc1 and MvSrc2 showed no discernible preference for a peptide substrate that contains an SH2 ligand (pYEEI). This pattern of substrate targeting is similar to that of the Src family kinases from *M. brevicollis* and *Capsaspora*.[12,17] The lack of SH2-dependent substrate targeting could potentially be due to an inability of the MvSrc1 and MvSrc2 SH2 domains to bind phosphotyrosine. To test whether MvSrc1 and MvSrc2 have the capacity to bind pTyr, we performed an *in vitro* binding assay. We immobilized a pTyr-containing synthetic peptide on Affi-Gel resin and incubated the resin with MvSrc1, MvSrc2, or *M. brevicollis* MbSrc1. (We previously showed that the binding affinity and selectivity of the MbSrc1 SH2 domain was similar to that of mammalian c-Src.[12]) After washing the samples, we measured binding by Western blotting (Figure 4C). Each Src kinase bound selectively to the immobilized pTyr-containing peptide, suggesting that the SH2 domains of MvSrc1 and MvSrc2 are functional.

Autophosphorylation on the activation loop is a hallmark of Src family kinases.[7,26,30] In mammalian Src, this modification increases kinase activity by destabilizing the autoinhibited conformation. To test for autophosphorylation of MvSrc1 and MvSrc2, we first dephosphorylated the purified proteins by treating them with YOP tyrosine phosphatase. We then incubated the kinases with [γ-^{32}P]ATP and analyzed the reactions by SDS−PAGE and autoradiography. Autophosphorylation of MvSrc1 and MvSrc2 was readily detected after 5 min (Figure 5A). To measure stoichiometry, we excised the protein bands and analyzed them by scintillation counting. Under these conditions, MvSrc1 incorporated 0.46 mol of phosphate/mol of enzyme and MvSrc2 incorporated 0.13 mol/mol (Figure 5B). We also tested the effect of autophosphorylation on enzymatic activity, using a synthetic peptide substrate. Dephosphorylated MvSrc1 and MvSrc2 showed background levels of activity, while the ATP-treated enzymes were active (Figure 5C).

MvSrc1 and MvSrc2 Are Inactive in Mammalian Cells. The unicellular homologues of c-Src show variable levels of activities when expressed heterologously in mammalian cells. *M. brevicollis* MbSrc1 is active in mammalian cells, and it can functionally replace c-Src in a transcriptional activation assay based on phosphorylation of Stat proteins.[8,12] *Capsaspora* CoSrc2 phosphorylates itself and other proteins when expressed in mammalian cells, while CoSrc1 is inactive.[17] We expressed FLAG-tagged MvSrc1 and MvSrc2 in human embryonic kidney (HEK) 293T cells. Cells expressing the enzymes showed weak tyrosine phosphorylation of ≈125, ≈50, and ≈25 kDa proteins, but these proteins were also phosphorylated in untransfected 293T cells (Figure S3 of the Supporting Information). No significant autophosphorylation of MvSrc1 or MvSrc2 was observed in these experiments, despite robust expression of MvSrc1 and MvSrc2. We obtained similar results in Src/Yes/Fyn-deficient fibroblast cells (data

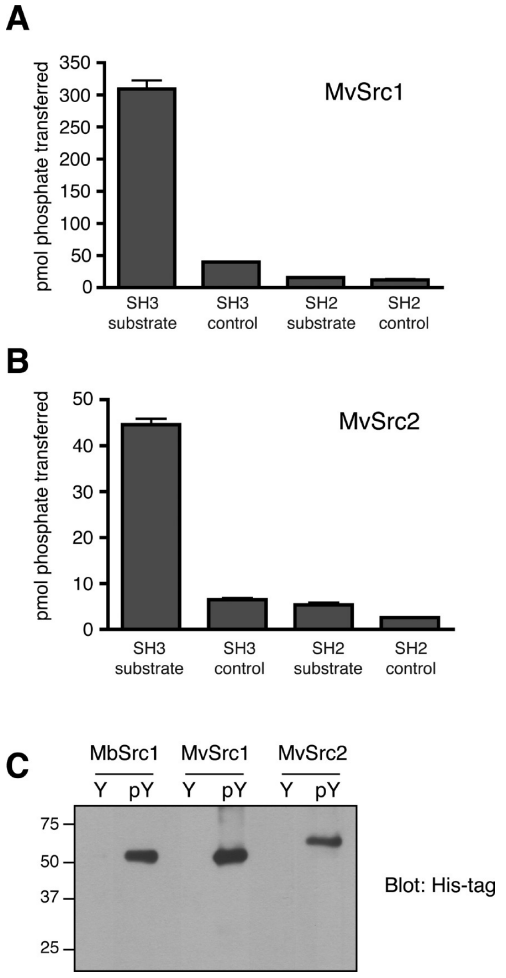

Figure 4. Substrate targeting. (A) MvSrc1 (1 μM) was tested with synthetic peptide substrates containing SH3 or SH2 ligand sequences or matched controls (100 μM). Reactions proceeded for 10 min at 30 °C and were analyzed using the phosphocellulose paper binding assay. (B) Similar experiments were conducted with MvSrc2 (5 μM). (C) Binding of MvSrc1, MvSrc2, and *Monosiga* MbSrc1 to an immobilized SH2 ligand. Enzymes (500 nM) were incubated with Affi-gel 15 resins containing either the EPQpYEEIPIKQ peptide [12] (pY) or the unphosphorylated peptide (Y) in buffer containing 50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100. After 45 min, the samples were centrifuged and washed four times with binding buffer. Proteins were eluted by incubation with SDS−PAGE sample buffer. Bound proteins were visualized by Western blotting with the anti-His tag antibody.

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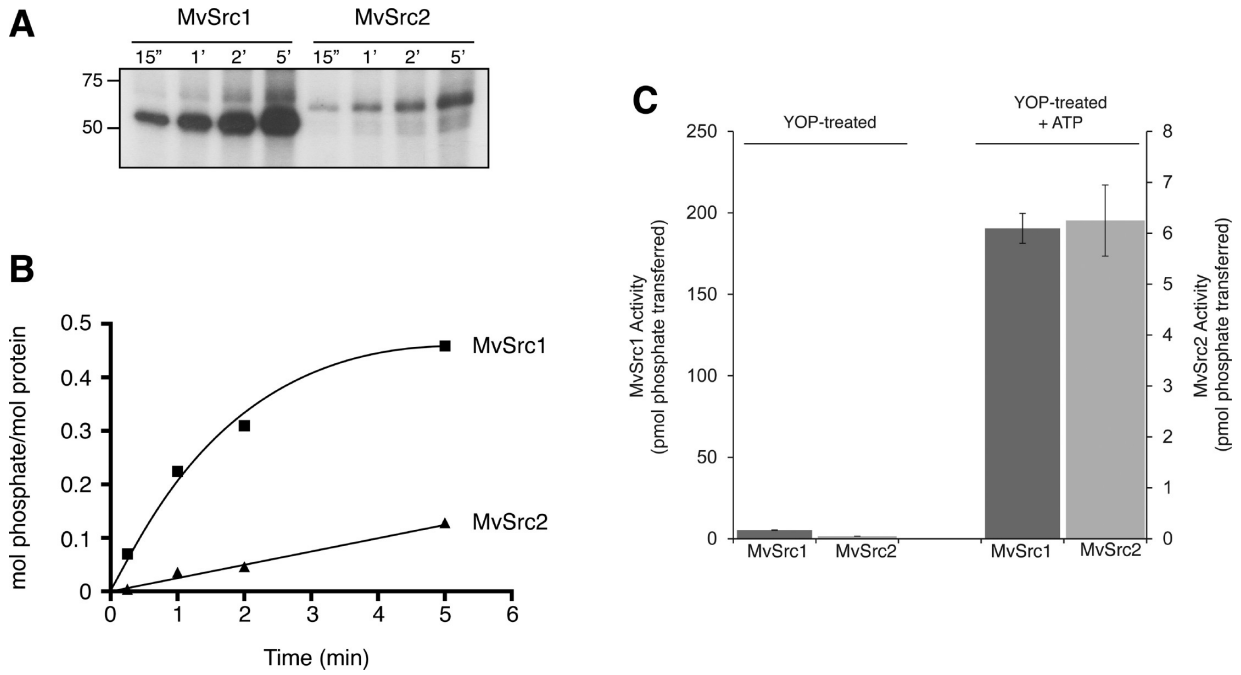

Figure 5. Autophosphorylation of MvSrc1 and MvSrc2. (A) Enzymes (1 μM) were treated with immobilized GST-YOP phosphatase for 30 min at 30 °C. The YOP was removed by centrifugation, and the enzymes were incubated with [γ-^{32}P]ATP. Samples were removed at the indicated time points and reactions stopped by mixing the samples with SDS−PAGE sample buffer. The reactions were analyzed by SDS−PAGE and autoradiography. (B) Bands from the gel shown in panel A were excised, and the stoichiometry of autophosphorylation was measured by scintillation counting. (C) MvSrc1 and MvSrc2 were treated with immobilized GST-YOP phosphatase for 30 min at 30 °C. The YOP was removed by centrifugation, and the samples were assayed directly or after incubation with 0.5 mM ATP for 30 min at 30 °C. Activity measurements were performed with the Src synthetic peptide substrate and the phosphocellulose paper assay. Note that two different activity scales were used for MvSrc1 and MvSrc2.

not shown). It is possible that the N-terminal FLAG tags of MvSrc1 and MvSrc2 interfere with subcellular localization and substrate recognition, although an N-terminally FLAG-tagged version of mammalian c-Src showed robust activity in these experiments (Figure S3 of the Supporting Information).

Cloning *Mi. vibrans* MvCsk. A C-terminal Src kinase (Csk) homologue was cloned by PCR. MvCsk possesses the conserved SH2, SH3, and kinase domain arrangement seen in metazoan, choanoflagellate, and *Capsaspora* Csk proteins (Figure 6 and Figure S4 of the Supporting Information). The

Percent amino acid identity

Sponge Csk	68.6	61.2	57.0
Xenopus Csk	77.1	89.3	84.6
Capsaspora CoCsk	31.4	47.7	51.2
Ministeria MvCsk	40.0	48.3	52.2

Figure 6. Homology between human Csk and unicellular Csks. The Csk domain structure is shown schematically. The percent amino acid identity among the SH3, SH2, and kinase domains of human Csk and various unicellular Csk kinases are shown below the structure.

amino acid sequence of the kinase domain of MvCsk is 52.2% identical with that of the human Csk kinase domain, and the sequences of SH3 and SH2 domains are 40 and 48.3% identical with those of human Csk (Figure 6). A multiple-sequence alignment of MvCsk with homologues from humans, *Xenopus* (frog), *Ephydatia fluviatilis* (a simple sponge), *M. brevicollis*, and *Capsaspora* shows that MvCsk contains the key catalytic residues, and all six residues previously identified as being important for mammalian Src recognition[34] (Figure S4 of the Supporting Information).

MvCsk Has No Measurable Activity. We amplified the MvCsk cDNA from a *Ministeria* cDNA library by PCR. We cloned full-length MvCsk into a bacterial expression vector and produced the kinase as a fusion protein with glutathione *S*-transferase (GST). After purification of GST-tagged MvCsk protein by glutathione-agarose chromatography, we assayed activity with a generic tyrosine kinase substrate, the synthetic peptide poly(Glu_4-Tyr). No activity could be detected for MvCsk, in contrast to mammalian Csk and *M. brevicollis* MbCsk, which were produced under similar conditions in bacteria (Figure 7A). The lack of MvCsk activity was similar to results obtained previously for Csk from the related filasterean *Capsaspora*.[17] Next, we tested the activity of MvCsk toward purified MvSrc1 and MvSrc2 in an *in vitro* reaction with [γ-^{32}P]ATP. As a positive control, we conducted a parallel reaction with GST-tagged mammalian Csk and a kinase dead form of c-Src. No MvCsk activity toward the *Ministeria* Src proteins was detectable, although mammalian Csk phosphorylates c-Src under these conditions (Figure 7B). Mammalian Csk was unable to phosphorylate MvSrc1 or MvSrc2 (data not shown). Expression of MvCsk in HEK293T cells resulted in no tyrosine phosphorylation of MvSrc1, MvSrc2, or other cellular proteins (Figure S5 of the Supporting Information).

Mammalian Csk phosphorylates the C-terminal tail of Src, stabilizing the autoinhibited form and reducing its kinase activity.[7,26] Although MvCsk did not display any activity toward a general tyrosine kinase substrate or MvSrc, we tested its ability to regulate MvSrc activity. MvSrc1 and MvSrc2 were incubated with MvCsk *in vitro*, and MvSrc activity was

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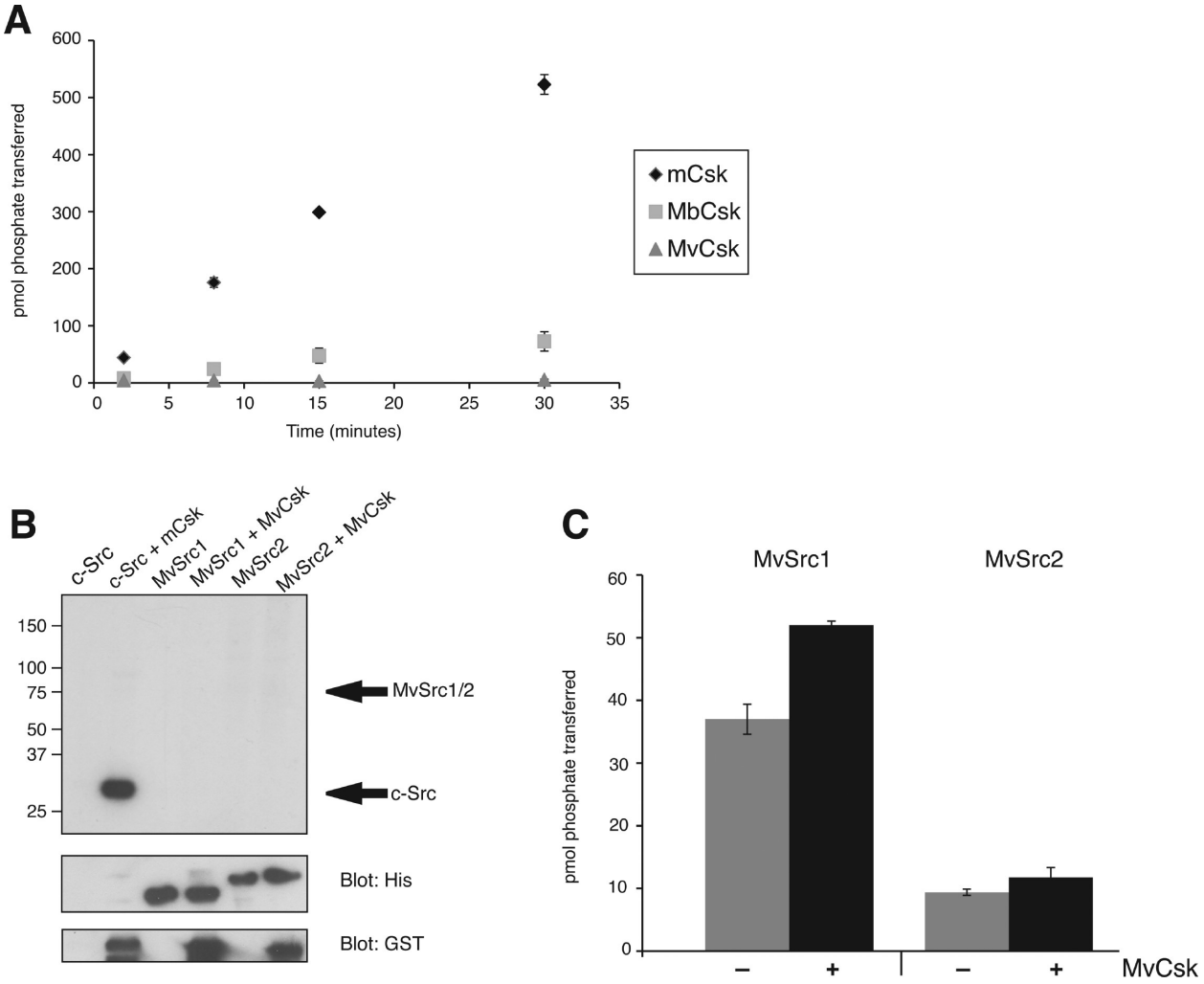

Figure 7. MvCsk lacks kinase activity. (A) Purified MvCsk, *M. brevicollis* MbCsk, and mammalian Csk (mCsk) (200 nM) were assayed with poly(Glu_4-Tyr) (1 mg/mL). The reaction mixtures contained 0.5 mM [γ-^{32}P]ATP. Activity was analyzed at various time points by scintillation counting. (B) Purified MvCsk (1 μM) was incubated with MvSrc1 or MvSrc2 (1 μM) in the presence of [γ-^{32}P]ATP. Prior to the MvCsk reactions, MvSrc1 and MvSrc2 were pre-autophosphorylated with 1 mM unlabeled ATP for 1 h. A positive control reaction was conducted with mammalian Csk and a kinase inactive mutant form of the c-Src kinase domain (with the C-terminal tail). The reactions were stopped after 30 min by addition of SDS–PAGE sample buffer and analyzed by autoradiography. Aliquots were also analyzed by Western blotting to confirm the presence of MvSrc1 and MvSrc2 (anti-His tag) and Csks (anti-GST). (C) MvCsk does not inhibit MvSrc. *In vitro* MvSrc1 and MvSrc2 reactions were performed in the presence or absence of MvCsk. Reaction mixtures contained 1 μM enzymes, 0.5 mM [γ-^{32}P]ATP, and 0.4 mg/mL RCM-lysozyme as a Src substrate. After 20 min at 30 °C, reaction mixtures were analyzed by scintillation counting.

measured in a radioactive kinase assay (in control experiments, we confirmed that MvCsk itself had no activity toward the Src substrate peptide). No inhibition of MvSrc1 or MvSrc2 was observed in the presence of MvCsk; instead, MvSrc activity was modestly increased by MvCsk treatment (Figure 7C). Similar results were obtained previously for the *C. owczarzaki* Src and Csk enzymes.[17]

Molecular Basis for the Lack of Activity in Filasterean Csk Kinases. Previous structural and biochemical studies of mammalian Csk kinase have highlighted their significant differences from Src family kinases. The isolated tyrosine kinase catalytic domain of Csk has low activity.[35] In contrast to the autoinhibited structure of Src, the Csk SH3 and SH2 domains bind to the kinase N-lobe to stabilize an active conformation.[36] The β3–αC loop in the Csk kinase domain interacts with the SH2 domain, and mutations in this interface decrease Csk activity.[37] The SH3–SH2 linker region is also important for Csk activity.[38]

The availability of two inactive filasterean Csk kinases (from *Capsaspora* and *Ministeria*) allows a comparison with multiple active Csk enzymes, including those from mammals, sponge (a simple metazoan), and the unicellular choanoflagellate *M. brevicollis*. The sequences of CoCsk and MvCsk were strongly homologous to the sequences of their counterparts in mammalians and choanoflagellates, and the important residues for ATP binding and catalysis are conserved in the kinase domain (Figure S4 of the Supporting Information). On the other hand, several residues in the SH2–kinase interface and the SH3–SH2 linker of CoCsk and MvCsk diverge from the sequences of the active enzymes (Figure 8A). In particular, CoCsk and MvCsk contain a glycine residue at position 236 (MvCsk numbering), but in mammalian, *Xenopus*, sponge, and *M. brevicollis* Csk, this residue is an asparagine or aspartic acid. In mammalian Csk, substitution of this residue with alanine led to a loss of kinase activity.[38] To test the importance of this residue in MvCsk, we introduced the Asn residue found in

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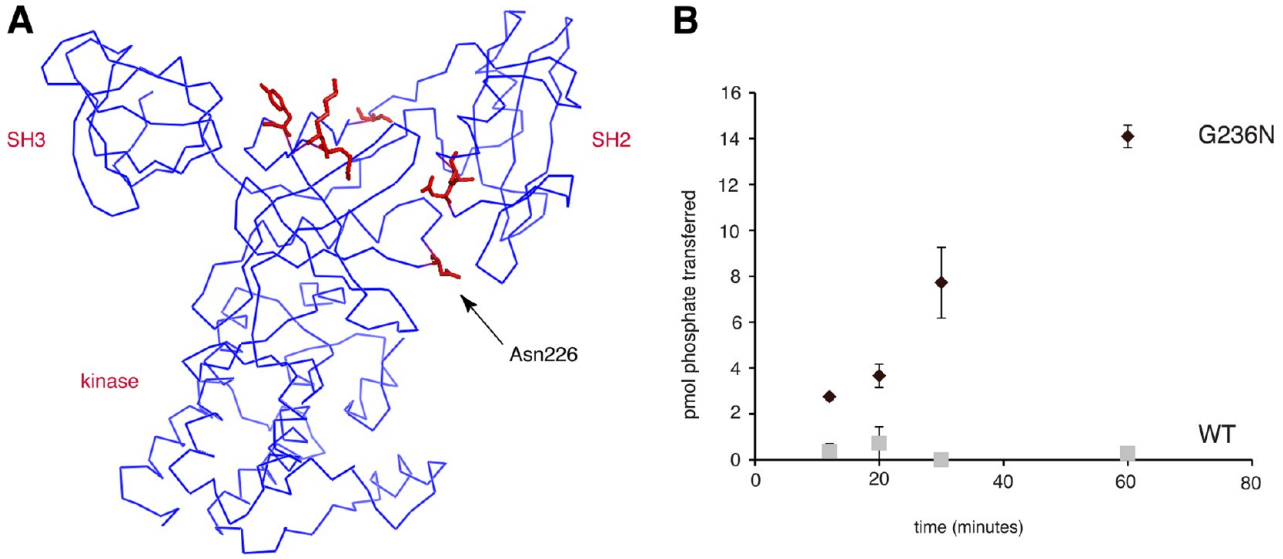

Figure 8. G236N mutation restores MvCsk activity. (A) The structure of rat Csk (Protein Data Bank entry 1K9A). Amino acids colored red are residues in the SH2−kinase interface that are conserved between *Capsaspora* and *Ministeria* Csks but diverge from active Csks. The position of N226 in rat Csk (at the position corresponding to MvCsk G236) is indicated by an arrow. This figure was prepared with PyMOL. (B) Purified wild-type GST-MvCsk (gray squares) and GST-MvCsk-G236N (black diamonds) were assayed with poly(Glu_4-Tyr) (1 mg/mL). The reaction mixtures contained 0.5 mM [γ-^{32}P]ATP. Activity was analyzed at various time points by scintillation counting.

mammalian Csk. We produced a G236N mutant form of MvCsk, expressed and purified the GST fusion protein, and tested the activity against a synthetic substrate peptide. The mutation caused a partial recovery of MvCsk activity, as measured toward poly(Glu_4-Tyr) (Figure 8B). The level of activity for MvCsk-G236N was 19% of the activity of a similar preparation of *M. brevicollis* MbCsk or 3% of the activity of mammalian Csk. No activity was detectable toward MvSrc1, MvSrc2, or the Src substrate peptide (data not shown). These results reinforce the importance of SH2−kinase domain interactions in Csk activity and suggest an explanation for the low Csk activity observed in filastereans.

DISCUSSION

Filastereans, the sister group to metazoans and choanoflagellates, consist of two known species: *C. owczarzaki* and *Mi. vibrans*. Genomic analysis of *Capsaspora* identified 103 putative tyrosine kinase genes, of which 92 are predicted to be receptor TKs. The 11 remaining TKs are predicted to be nonreceptor-type kinases.[5] A PCR-based survey of TKs in *Ministeria* showed that the repertoire of nonreceptor TKs is similar in the two filasterean species.[5] Of the 10 common families of metazoan nonreceptor TKs, six are present in at least one filasterean. In particular, the common domain architecture of the Src, Csk, Tec, and Abl families (SH3−SH2−kinase) was established before the divergence of filastereans from choanoflagellates and metazoans. This domain arrangement is conserved throughout metazoans,[9,39] and experimental alterations to the domain structure lead to improper regulation and alterations in substrate recognition.[40]

Here, we have focused on Src family tyrosine kinases. In multicellular animals, Src kinases play important roles in the regulation of cell growth and proliferation, survival, adhesion, and migration.[7,30] Inappropriate expression of activated Src (as in cells transformed by the viral oncogene v-Src) leads to increased motility, uncontrolled proliferation, and anchorage independence. Thus, the activity of Src family kinases must be tightly regulated. Phosphorylation of the C-terminal negative regulatory tyrosine (Y527) by Csk is a unique aspect of Src family kinases that has been conserved throughout the evolution of metazoans. When the Csk gene is knocked out in mice, Src is hyperactivated and early stage embryonic death occurs.[41]

Although Csk-mediated Src negative regulation is essential in metazoans, studies of ancestral forms of Src have shown that Src can operate without strict control in protists. The Csk homologues from the choanoflagellates *M. ovata* and *M. brevicollis* are able to phosphorylate their cognate Src enzymes, but they do not impose negative regulation.[12,13] In *C. owczarzaki*, the closest relative to metazoans and choanoflagellates, Src is enzymatically functional yet Csk lacks any detectable catalytic activity.[17] In this report, we have studied the Src−Csk regulatory pair in *Mi. vibrans*, an organism in the same phylogenetic group as *Capsaspora*. *Ministeria* Src1 and Src2 are active tyrosine kinases (Figure 3). MvSrc1 had an approximately 10-fold higher specific activity than MvSrc2 toward a synthetic peptide containing an optimal Src recognition sequence. Both enzymes phosphorylated an SH3 ligand-containing peptide at a rate higher than that of a control peptide but did not show a preference for a peptide with an SH2 ligand (Figure 4A). These results mirror those obtained for *C. owczarzaki* CoSrc1 and CoSrc2 and *M. brevicollis* MbSrc1, strengthening the idea that the coupling of the SH2 and kinase domains developed later in SFK evolution.

An unexpected observation was that MvSrc2 has significant Ser/Thr kinase activity (Figure 3). Moreover, the peptide phosphorylated by MvSrc2 (Kemptide) lacks the acidic residues commonly found in TK substrates. The sequences surrounding the presumed autophosphorylation sites in the activation loops of MvSrc1 and MvSrc2 differ. MvSrc1 contains the sequence LITDDEY, while MvSrc2 has the sequence IITKNDGAY (Figure S1 of the Supporting Information). Thus, MvSrc2 may be less dependent on acidic residues as specificity determinants than other TKs. TKs normally show high specificity for phosphorylating tyrosine residues, while

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other eukaryotic protein kinases target serine and threonine residues.[42] It has been proposed that primitive enzymes in general had broader specificities than modern enzymes, allowing fewer enzymes to carry out the processes needed for ancestral organisms.[43] Some modern protein kinases, such as MEK1, MEK2, and Wee1, designated dual-specificity kinases, have the ability to phosphorylate both serine and tyrosine residues.[44,45] These enzymes usually have relatively narrow substrate specificities, and their amino acid sequences more closely resemble those of Ser/Thr kinases than those of TKs. It has been suggested that dual-specificity kinases served as an intermediary step between the more ancient Ser/Thr kinases and TKs: after duplication of dual-specificity kinase genes, the new enzymes lost the ability to phosphorylate serine and threonine, leading to the birth of the new tyrosine kinase subgroup.[10,46,47] MvSrc2 may therefore represent an intermediary form of a tyrosine kinase; it is a tyrosine kinase by amino acid sequence homology, yet it is able to act as a dual-specificity kinase. Additional studies are needed on the Src-like kinases of earlier ancestors (e.g., ichthyosporeans) to test for the presence of dual specificity or determine if the phenomenon is specific to MvSrc2.

We previously reported that the *C. owczarzaki* homologue of Csk (CoCsk) lacked detectable enzymatic activity, in contrast to all of the previously studied Csk proteins.[17] Here, we show in the filasterean *Mi. vibrans* that MvCsk is inactive toward a general TK substrate or MvSrc proteins (Figure 7). MvCsk was unable to inhibit MvSrc; treatment with MvCsk actually caused a slight increase in MvSrc activity (Figure 7C), perhaps because of the disruption of an autoinhibitory interaction. The availability of the two inactive filasterean Csk kinases has allowed us to identify a region (the SH2−kinase interface) that is a critical determinant of Csk enzymatic activity. A mutation in this region (G236N) partially restored MvCsk activity (Figure 8B). These results help to illuminate the evolutionary history of Csk activity. Csk may have evolved as an active enzyme that lost its activity in the filasterean lineage. Alternatively, MvCsk and CoCsk may have emerged initially as pseudokinases and gained catalytic activity after the split between filastereans and choanoflagellates. In this view, the original role of MvCsk and CoCsk would have been similar to those of other pseudokinases (scaffolding, activating other proteins through domain binding, or targeting of active proteins to specific cellular locations).[48,49] Csk may have been subsequently co-opted in metazoans to provide negative regulation of Src family kinases. A more complete understanding of the physiological roles of MvSrc1, MvSrc2, and MvCsk in intact *Mi. vibrans* cells awaits the development of experimental tools to manipulate gene function in filastereans.

■ ASSOCIATED CONTENT

Supporting Information

Five supplemental figures and legends. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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