Functional development of Src tyrosine kinases during evolution from a unicellular ancestor to multicellular animals

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The Src family of tyrosine kinases play pivotal roles in regulating cellular functions characteristic of multicellular animals, including cell–cell interactions, cell-substrate adhesion, and cell migration. To investigate the functional alteration of Src kinases during evolution from a unicellular ancestor to multicellular animals, we characterized Src orthologs from the unicellular choanoflagellate *Monosiga ovata* and the primitive multicellular sponge *Ephydatia fluviatilis*. Here, we show that the *src* gene family and its C-terminal Src kinase (Csk)-mediated regulatory system already were established in the unicellular *M. ovata* and that unicellular Src has unique features relative to multicellular Src: It can be phosphorylated by Csk at the negative regulatory site but still exhibits substantial activity even in the phosphorylated form. Analyses of chimera molecules between *M. ovata* and *E. fluviatilis* Src orthologs reveal that structural alterations in the kinase domain are responsible for the unstable negative regulation of *M. ovata* Src. When expressed in vertebrate fibroblasts, *M. ovata* Src can induce cell transformation irrespective of the presence of Csk. These findings suggest that a structure of Src required for the stable Csk-mediated negative regulation still is immature in the unicellular *M. ovata* and that the development of stable negative regulation of Src may correlate with the evolution of multicellularity in animals.

choanoflagellate | Csk | sponge

The Src family of tyrosine kinases originally were identified as the first proto-oncogene products (1, 2), and are known to play pivotal roles in the regulation of diverse cellular functions characteristic of multicellular animals (3). Src kinase activity is involved in the regulation of a variety of cell-adhesion molecules (4–6). Tyrosine phosphorylation of catenin family proteins by activated Src leads to the disruption of cadherin-mediated cell–cell interactions (7–9), which confers metastatic potential on some cancer cells (10, 11). Activation of integrins induces activation of Src-mediated pathways involved in turnover of focal adhesions and cell migration (12–14). Cell–cell interactions mediated by the Ig superfamily proteins also are regulated by Src-mediated pathways (15). Furthermore, the organization of specialized cell layers in the nervous system is controlled by Src family kinases (16, 17). These lines of evidence suggest that Src family kinases are indispensable for the development and maintenance of multicellular organisms.

Src family kinases are strictly regulated by environmental cues; activity is regulated positively by autophosphorylation at a tyrosine in the catalytic pocket (Tyr-416 in chicken c-Src) and negatively by phosphorylation of a C-terminal tyrosine (Tyr-527 in chicken c-Src; refs. 3 and 18). This mode of negative regulation is a hallmark of Src kinases: No other tyrosine kinases are regulated negatively through tyrosine phosphorylation. Phosphorylation of the negative regulatory site is catalyzed by the C-terminal Src Kinase (Csk; ref. 19), and the mechanism has been elucidated at the molecular level (20, 21). Intramolecular binding of the phosphorylated C-terminal tyrosine to the Src homology (SH)2 domain stabilizes interactions between the SH2, SH3, and SH2-kinase linker domains, thereby locking the catalytic pocket into a fully assembled inactive conformation. Upon stimulation by extracellular cues, Src family kinases become activated through interaction with adaptor proteins or by dephosphorylation of the regulatory site, enabling the opening of the inactive conformation (22). Csk loss-of-function in mice leads to constitutive activation of the Src family kinases, accompanied by severe developmental defects (23, 24), indicating that Csk-mediated negative regulation of Src is essential for normal development in animals. Furthermore, it has been shown that the Src/Csk regulatory system has been highly conserved throughout animal evolution and arose early in metazoan evolution (25–27). These observations demonstrate that Src family kinases can function in multicellular animals under the stringent control of Csk. However, critical questions remain to be answered: why Src acquired a Csk-mediated negative regulation system and how the function of Src developed during animal evolution, particularly at the critical transition from unicellular ancestor to multicellular animal.

Choanoflagellates are the group of unicellular or colonial protists most closely related to animals and have been used as a model for studies of early animal evolution (28–30). Comparative gene expression studies have shown that choanoflagellates possessed genes encoding cell-adhesion proteins and cell-signaling molecules, which were thought to be restricted to multicellular animals (31, 32). Despite being unicellular, choanoflagellates express cadherin-like and C type lectin-like molecules that directly mediate cell–cell interactions. Furthermore, a variety of signaling molecules located downstream of such adhesion proteins, including Src, Ras, phosphatidylinositol 3-kinase, and Raf, also are expressed in these unicellular protists. These findings suggest that the molecules involved in multicellularity evolved before the origin of animals and were later co-opted for animal development. However, how these molecules were co-opted or evolved novel functions during the transition from unicellular to multicellular life remains completely unknown.

To investigate the functional development of Src and Csk during the critical transition of animal evolution, we isolated potential orthologs of Src and Csk from a unicellular protist, the choanoflagellate *Monosiga ovata* (33), and a primitive multicellular

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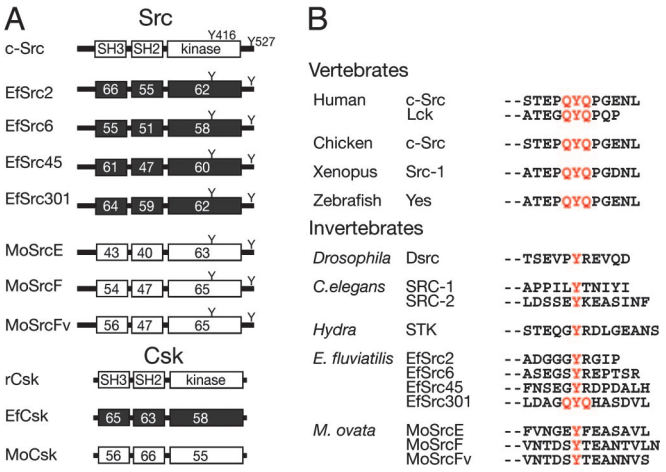

Fig. 1. Schematic of Src and Csk ortholog structures and C-terminal sequence alignments. (*A*) Structures of Src and Csk orthologs from *E. fluviatilis* and *M. ovata*. SH3, SH2, and kinase domains are boxed. Numbers in the boxes denote percent amino acid identity to chicken c-Src. An autophosphorylation site (Y416) and the C-terminal regulatory site (Y527) are indicated by Ys. Accession numbers of the sequences and alignments of the deduced amino acid sequences of Src and Csk orthologs are shown in Figs. 6 and 7, respectively. (*B*) C-terminal sequence alignments of Src relatives from the species indicated. Csk-phosphorylated tyrosine residues (Y) and adjacent Gln (Q) residues are shown in red.

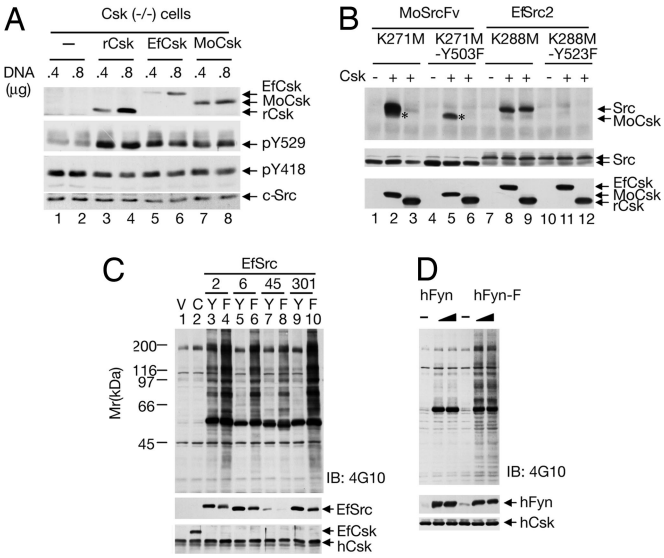

Fig. 2. EfCsk and MoCsk activity and regulation of EfSrc. (*A*) Expression vectors for rat Csk (rCsk), EfCsk, or MoCsk were transfected into Csk-deficient [Csk(−/−)] cells (35) at the concentrations indicated. Total cell lysate was analyzed by Western blotting with anti-Csk (for rCsk and EfCsk), anti-Myc (for MoCsk), anti-Src pY529, anti-Src pY418, or anti-Src. (*B*) Specificities of EfCsk and MoCsk. A kinase-deficient MoSrcFv (K271M), a double mutant MoSrcFv having an additional substitution at Tyr-503 (K271M-Y503F), a kinase-deficient EfSrc2 (K288M), and a double mutant EfSrc2 (K288M-Y523F) were expressed in *S. cerevisiae* with or without the Csk ortholog indicated. Total cell lysates were subjected to Western blotting by using anti-phosphotyrosine 4G10 (*Top*), anti-FLAG (for Src, *Middle*), or anti-Myc (for Csk, *Bottom*). Asterisks indicate autophosphorylated MoCsk. (*C*) EfSrc isoforms or their YF C-terminal mutants were transiently expressed in 293T cells, and total cell lysates were analyzed by Western blotting with 4G10, anti-Myc (for EfSrc), anti-Csk, and anti-Src. V, empty vector; C, Csk expression vector; Y, wild-type Src; F, YF mutant Src; 2, EfSrc2; 6, EfSrc6; 45, EfSrc45; 301, EfSrc301. (*D*) Increasing amounts (0, 0.25, or 0.5 μg) of vectors expressing hFyn and hFyn-F were transfected transiently into 293T cells. Total cell lysates were analyzed by Western blotting.

animal, the freshwater sponge *Ephydatia fluviatilis* (34), and compared their biochemical features, focusing in particular on regulatory systems.

Results

Cloning of Src and Csk from *E. fluviatilis* and *M. ovata*. Full-length cDNAs encoding tyrosine kinases were obtained from *E. fluviatilis* and *M. ovata* by PCR-based cloning (27, 34). Potential Src orthologs were selected based on the presence of characteristic structural domains shared by all Src family members. These domains occur in a defined order: an N-terminal fatty acylation signal; SH3 and SH2 domains; a tyrosine kinase domain with an autophosphorylation site in the activation loop; and a C-terminal regulatory tyrosine (2). Four clones from *E. fluviatilis* (EfSrc2, 6, 45, and 301) and three from *M. ovata* (MoSrcE, F, and Fv) were identified as potential Src orthologs (Fig. 1*A*). Phylogenetic analysis classified these EfSrc and MoSrc into the Src subgroup of tyrosine kinases (Fig. 6, which is published as supporting information on the PNAS web site).

Alignment of these Src orthologs revealed that the kinase domains are highly conserved, with amino acid identities to chicken c-Src ranging from 58% to 62% (EfSrc) and from 63% to 65% (MoSrc) (Fig. 1*A*). The C-terminal regulatory domains of these primitive Src orthologs were aligned with those of other species (Fig. 1*B*). The regulatory sequences are highly conserved among vertebrate Src orthologs, but invertebrate sequences (including those of *E. fluviatilis* and *M. ovata*) are more divergent. Interestingly, EfSrc301 has a QYQ motif that is conserved in vertebrate Src, although adjacent sequences are not conserved. In addition, critical amino acid residues required for intramolecular domain interactions (Y90, Y135, and P250 in chicken c-Src) all are conserved in EfSrc and MoSrc (Fig. 7, which is published as supporting information on the PNAS web site), suggesting that these primitive Src relatives may be regulated in a manner similar to vertebrate Src. These findings demonstrate that *src* genes already were established in the common unicellular ancestor of choanoflagellates and animals and were duplicated before the separation of the two lineages.

We also identified one Csk ortholog from each organism (named EfCsk or MoCsk) based on the characteristic features of the SH2, SH3, and tyrosine kinase domains and the absence of a fatty acylation signal, autophosphorylation site, or regulatory site (Fig. 1*A*). Like vertebrate Csk, only a single Csk so far has been identified from each species, suggesting that the Src/Csk regulatory unit, comprising multiple Src isoforms and a single Csk, predates the development of animals.

Functions of Csk from *E. fluviatilis* and *M. ovata*. To examine whether the Src/Csk regulatory system is functional in *E. fluviatilis* and *M. ovata*, we first analyzed the activity of Csk orthologs by using a Csk-deficient mouse cell line (35). In these cells, rat Csk (rCsk), EfCsk, and MoCsk phosphorylated the C-terminal tyrosine of mouse c-Src and suppressed its activity, as evidenced by increased phosphorylation at Y529 and decreased phosphorylation at Y418 (Fig. 2*A*). In a yeast expression system (Fig. 2*B*), MoCsk was able to phosphorylate kinase-deficient MoSrcFv (K271M) but not its mutant having a Tyr to Phe substitution at the C-terminal tyrosine (K271M-Y503F). These results indicate that MoCsk phosphorylates Y503 of MoSrcFv and EfCsk phosphorylates Y523 of EfSrc2 specifically. However, rCsk phosphorylated EfSrc efficiently but MoSrcFv only poorly. These observations demonstrate that MoCsk and EfCsk are functional orthologs of Csk and that both Src and Csk functions are, at least partially, complementary between *E. fluviatilis*, *M. ovata*, and vertebrates.

Regulatory Features of *E. fluviatilis* Src. Csk regulation of EfSrc was analyzed by using a transient expression system in 293T cells, in which the endogenous human Csk is sufficient for phosphorylation

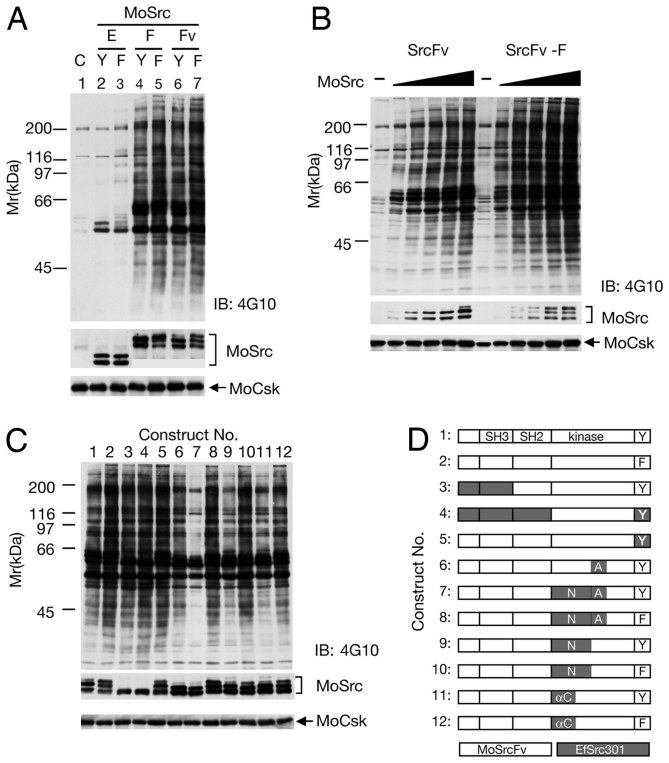

Fig. 3. Regulatory features of MoSrc. (*A*) MoSrc isoforms or their YF C-terminal mutants were coexpressed with MoCsk in 293T cells, and total cell lysates were analyzed by Western blotting with 4G10, anti-Myc (for MoSrc), or anti-HA (for MoCsk). (*B*) Dose dependency of MoSrcFv activity. 293T cells were cotransfected transiently with a fixed amount of vector expressing MoCsk (0.5 μg) and increasing amounts of vectors (0–1.0 μg) expressing MoSrcFv or their YF mutants. Total cell lysates were analyzed by Western blotting. (*C*) The chimeric Src molecules consisting of domains from MoSrcFv and EfSrc301 were coexpressed transiently with MoCsk in 293T cells. Total cell lysates were analyzed by Western blotting for the proteins indicated. (*D*) Structures of the chimeric Src molecules. Construct numbers correspond to lane numbers in *C*. A, activation loop; N, N lobe of the kinase domain; αC, N-terminal half of the N lobe containing the αC helix. Detailed maps of the regions substituted in these chimeras are shown in Fig. 7.

of EfSrc. Activities of EfSrc isoforms were assessed by detecting tyrosine phosphorylation of 293T cellular proteins. Expression of EfSrc2 increased phosphorylation to a degree, whereas expression of its Y523F C-terminal mutant greatly enhanced phosphorylation levels (Fig. 2*C*, lanes 3 and 4). The other wild-type EfSrc isoforms (EfSrc6, 45, and 301) showed much less activity than EfSrc2, but their YF mutants expressed greater activities (lanes 5–10). As has already been established, the activities of vertebrate Fyn (Fig. 2*D*) and c-Src were completely repressed by Csk. These results show that EfSrc is negatively regulated by Csk phosphorylation in a manner similar to that of vertebrate Src family kinases, although EfSrc2 is relatively resistant to the negative regulation.

Regulatory Features of *M. ovata* Src. The regulatory features of MoSrc isoforms were examined in 293T cells coexpressing MoCsk. MoSrcE and its YF mutant exhibited only low levels of activity, whereas MoSrcF and MoSrcFv showed substantial activity even in the presence of MoCsk; their YF mutants exhibited even greater activity (Fig. 3*A*). When MoSrcFv was expressed at lower levels, it exhibited significant activity in a dose-dependent manner, indicating that MoSrcFv is not activated via transphosphorylation caused by protein overexpression (Fig. 3*B*). Similar results were obtained when MoSrcF was expressed (data not shown). These observations raised the possibility that Csk-mediated negative regulation is unstable in MoSrc, although the C-terminal tyrosine still performs inhibitory function.

To examine which domain of MoSrc is responsible for its unstable negative regulation, we compared the regulatory features of chimeric MoSrcFv-containing EfSrc301 domains that are stably regulated (Fig. 3 *C* and *D*). When the N-terminal region (lane 3), the SH2 and SH3 domains (lane 4), and the C-terminal regulatory domain (lane 5) of MoSrcFv were replaced with those of EfSrc301, the MoSrcFv chimeras continued to be regulated inefficiently, suggesting that intramolecular domain interactions required for negative regulation do not affect the activity of MoSrcFv. Replacement of the activation loop (A; Fig. 3*C*, lane 6) slightly improved the efficiency of regulation, whereas the additional replacement of the N lobe of the kinase domain (NA; Fig. 3*C*, lanes 7 and 8) successfully conferred more stable regulation to the MoSrcFv chimera. The replacement of the N lobe alone (N; Fig. 3*C*, lanes 9 and 10) or the half of the N-lobe containing the αC helix (αC; Fig. 3*D*, lanes 11 and 12) partially restored the negative regulation. These results suggest that the intramolecular interaction mediated by the C-terminal phosphorylation is functional in MoSrcFv and that alterations in the kinase domain are more important for the unstable negative regulation of MoSrcFv.

C-Terminal Tyrosine Phosphorylation of *M. ovata* Src. To further evaluate the contribution of C-terminal phosphorylation to the unstable negative regulation of MoSrc, we examined the correlations of the kinase activities of MoSrcFv and its representative EfSrc301 chimera (Fig. 3*C*, lane 7; named Fv/301) with their C-terminal phosphorylation levels (Fig. 4*A*). When expressed in 293T cells, MoSrcFv showed strong activity that was almost comparable to that of the YF mutant, and its activity was only slightly repressed by MoCsk expression (Fig. 4*Aa*, lanes 2–4). In contrast, the activity of Fv/301 was substantially lower than that of the YF mutant and was further suppressed to nearly basal levels by MoCsk expression (lanes 5–7). The *in vivo* kinase activities of these Src molecules were confirmed further by detecting the phosphorylation of cortactin, an actin-binding protein that serves as a Src substrate (Fig. 4*Ad*). In these assays, the anti-cortactin pY421 antibody also was able to recognize wild-type MoSrcFv and Fv/301 but not their YF mutants. This cross-reactivity probably reflects the partial sequence similarity that exists between the anti-pY421 epitope and the sequences adjacent to the phoshorylated C-terminal tyrosine. The reactivity to MoSrc was abolished by a phosphopeptide containing the conserved C-terminal region of MoSrc (Fig. 4*Af*) but not by a nonphosphorylated peptide (Fig. 4*Ae*). These observations demonstrate that the anti-pY421 can be used to detect the C-terminal phosphotyrosine of MoSrc.

In the immunoprecipitates of MoSrcFv and Fv/301, C-terminal phosphorylation was detected even in the absence of MoCsk, although phosphorylation was enhanced by MoCsk expression (Fig. 4*Bc*). This result suggests that MoSrc is phosphorylated to some extent by endogenous Csk, although more efficient phosphorylation was achieved by MoCsk expression. Notably, apparent stoichiometry of the C-terminal phsophorylation of MoSrcFv was equivalent almost to that of Fv/301. The *in vitro* kinase activity next was determined by detecting phosphorylation of recombinant cortactin (Fig. 4*C*). Consistent with the *in vivo* results (Fig. 4*Aa*), the relative specific activity of MoSrcFv was only slightly suppressed by MoCsk. In contrast, the activity of Fv/301 was approximately half of that of its YF mutant in the absence of MoCsk, and it was further suppressed by MoCsk expression. The suppression paralleled the increase in the C-terminal phosphorylation levels induced by MoCsk expression. These results demonstrate that negative regulation of MoSrcFv could be made more stably by replacing its kinase domain and that the Csk-phosphorylated form of MoSrcFv could express substantial activity.

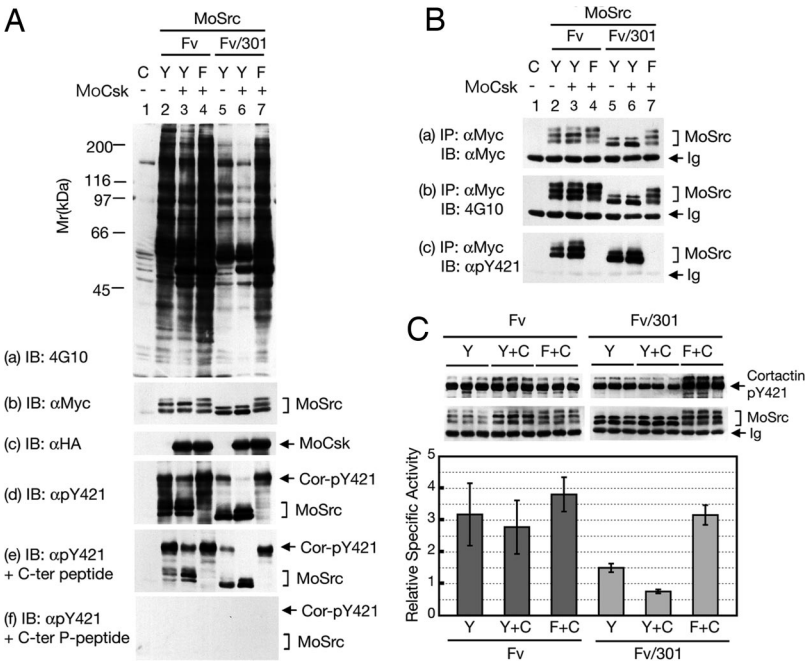

Fig. 4. Role of C-terminal tyrosine phosphorylation in the negative regulation of *M. ovata* Src. (*A*) MoSrcFv, the Fv/301 chimera or their YF C-terminal mutants were coexpressed with MoCsk in 293T cells. Total cell lysates were analyzed by Western blotting with the antibodies indicated. For the anti-pY421 competition assay, the antibody was preincubated with nonphosphorylated or phosphorylated peptides (20 μg/ml) corresponding to the C-terminal region of SrcFv. (*B*) MoSrc was immunoprecipitated from cell lysates by using anti-Myc and analyzed by Western blotting with the antibodies indicated. (*C*) The immunoprecipitates were subjected to *in vitro* kinase assays by using cortactin as a substrate. (*C Upper*) Tyrosine phosphorylation of cortactin and MoSrc levels were detected by Western blotting with anti-pY421 and anti-Myc, respectively (upper blot). Control immunorpecipitates gave no significant signals (data not shown). The results of triplicate assays are shown. The signal intensities for MoSrc (lower blot) and phosphorylated cortactin were quantified by using the Image J program (National Institutes of Health), and the relative specific activities were plotted (*C Lower*). Data represent means ± SD.

Functional Features of *M. ovata Src.* To evaluate the function of MoSrc, we compared rat embryonic fibroblast (REF-T) cells that stably expressed wild-type or YF mutants of MoSrcF and MoSrcFv with those expressing c-Src, c-SrcF527, v-Src, or Fv/301. In cells expressing MoSrc, tyrosine phosphorylation of cellular proteins was not suppressed significantly by MoCsk expression, even though the C-terminal tyrosine of MoSrc was highly phosphorylated (Fig. 8, which is published as supporting information on the PNAS web site). c-Src was regulated stringently by endogenous Csk, whereas c-SrcF527 and v-Src exhibited enormous activity. In cells expressing Fv/301, cellular tyrosine phosphorylation was suppressed by MoCsk expression, although the efficiency was lower than in 293T cells (Fig. 8).

The effects of MoSrc expression on cell morphology were observed (Fig. 5*A*). REF-T cells expressing MoSrcF or MoSrcFv tended to round up and occasionally showed a rod-like bipolar morphology similar to that of REF-T cells transformed by v-Src. In cells expressing MoSrcF or MoSrcFv, the morphological effects of MoCsk expression were not evident. In contrast, expression of MoCsk induced morphological changes in cells expressing Fv/301: The cells became flattened and the number of rounded-up cells was reduced greatly, although they continued to exhibit the depolarized phenotype. The transforming ability of these cells was examined by soft-agar colony formation assays (Fig. 5*B*). REF-T cells expressing v-Src formed a large number of colonies, as did those expressing MoSrcF, MoSrcFv, and Fv/301. Introduction of MoCsk into cells expressing MoSrcF or MoSrcFv had no significant effect on the number of colonies, whereas they were reduced significantly in cells expressing Fv/301. These results demonstrate that the activity of MoSrc is above the threshold required for cell transformation, irrespective of the presence of cognate Csk, and that alteration of MoSrc kinase domain could result in more stable Csk-mediated negative regulation.

Discussion

To investigate the molecular events that occurred during the transition from a unicellular ancestor to multicellular animals, we compared the regulatory features of Src-related tyrosine kinases isolated from multicellular sponge *E. fluviatilis* and a unicellular choanoflagellate *M. ovata*. We found that Src and the components required for its negative regulation, including its domain organization and functional Csk, were established in both *E. fluviatilis* and *M. ovata*. However, some Src relatives in these primitive organisms have not yet acquired stable negative regulation by Csk. Most of the EfSrc isoforms are stably regulated by Csk phosphorylation. However, one isoform (EfSrc2) retained some activity in the phosphorylated form, suggesting that it may represent an evolutionary intermediate or may play a specific role in particular cell types of *E. fluviatilis*. Among the three MoSrc isoforms, MoSrcF and MoSrcFv exhibit strong activity and low susceptibility to Csk-mediated negative regulation, whereas the third isoform (MoSrcE) shows only limited activity even in its YF mutant, implying that it may be a degenerate form. These differences in regulation and kinase activity of primitive Src relatives suggest that functional alterations in Src molecules occurred during the evolutionary transition.

Here we propose that structural alterations in the kinase domain are critical for the unstable negative regulation of MoSrc. However, the amino acid residues required for the establishment of negative regulation remain unidentified. In 293T cells, efficient negative regulation was achieved when the N lobe of the kinase domain was replaced with that of EfSrc301. The transforming assay using REF-T cells demonstrated that MoCsk provided efficient restoration of negative regulation to the Fv/301 chimera, further supporting the critical role of the N lobe of kinase domain in the negative regulation. In the N lobe of kinase domain, there are several amino acid residues that are not conserved between MoSrc and EfSrc. However, point mutations at potentially critical residues in EfSrc301 (A308K and Y382F) failed to attenuate its stable negative regulation (data not shown). These results suggest that the development of an overall kinase domain structure, which allows stable changes in the organization of the catalytic pocket, would be required for complete negative regulation. Alternatively, an unstable tertiary structure of the MoSrc kinase domain might enhance flexibility of the catalytic pocket, thereby increasing its basal activity. Additionally, it should be noted that the efficiency of negative regulation *in vivo* also may depend on the stability of the intramolecular interaction between SH2 and the C-terminal tyrosine and the turnover rate of C-terminal phosphorylation. To elucidate the precise molecular mechanism underlying regulation of these proteins, further structural analyses of primitive Src molecules will be required.

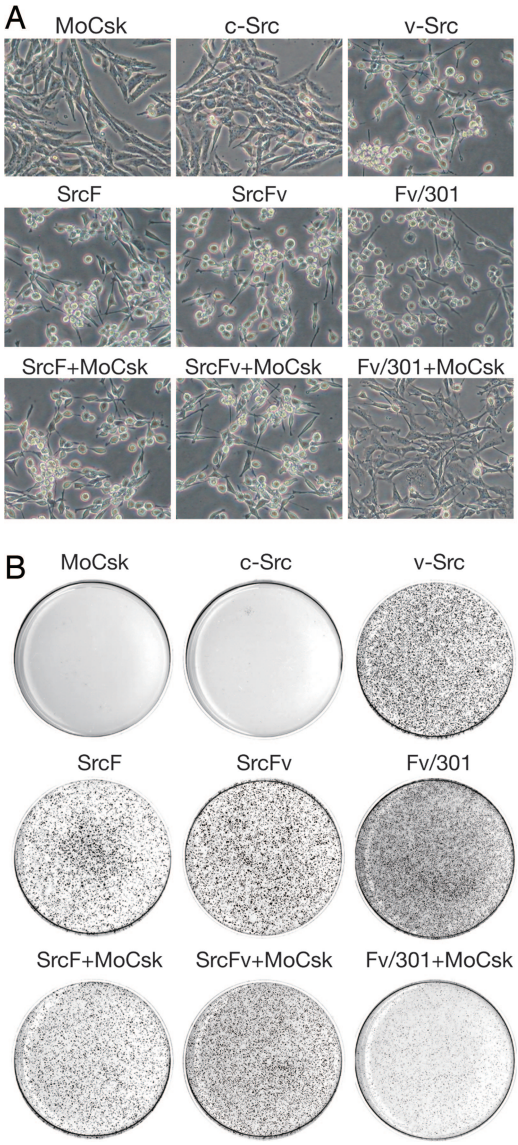

Fig. 5. Functional features of *M. ovata* Src. (*A*) Cell morphologies were observed under phase-contrast microscopy at a magnification of ×400. (*B*) Colony formation assay of the REF-T transfectants. Cells (4×10^4) were plated in soft agar, and after 5 d, colonies were stained with MTT reagent and photographed.

It has been shown that cell–cell interactions can be attenuated as a result of constitutive Src activation induced by the expression of a C-terminal YF mutant (36, 37) or a dominant negative form of Csk (38). Up-regulation of Src activity, caused by oncogenic mutations or overexpression in some cancers, leads to the loss of cell–cell adhesion and an increase in invasive or metastatic activity (10, 11). These observations reveal that the constitutive activity of Src has inhibitory effects on cell–cell interactions, and that negative regulation by Csk is critical for maintenance of cell–cell communication. In this study, we showed that Src relatives in the multicellular animal had established stable negative regulation, whereas those in the unicellular organism had not been fully developed. These results raise the possibility that there might be a correlation between the establishment of multicellular communication and the acquisition of stable negative regulation of Src during the evolution of multicellularity (Fig. 9, which is published as supporting information on the PNAS web site). To explore this hypothesis fully, more extensive comparative analyses will be required in other species of unicellular and multicellular organisms.

The unstable negative regulation of *M. ovata* Src suggests that this organism is generally exposed to high Src activity. Western blot analyses with specific antibodies to MoSrcF and MoSrcFv revealed that these Src proteins are indeed expressed (Fig. 10*A*, which is published as supporting information on the PNAS web site). Phosphoproteins corresponding to MoSrcF/Fv were detected with anti-pY421, suggesting that these Src proteins are phosphorylated at their C termini (Fig. 10*B*). Furthermore, it was shown that *M. ovata* ordinarily contains a variety of tyrosine phosphorylated proteins (Fig. 10*C*) and that phosphorylation of some proteins was potently inhibited by treatment with the Src inhibitor PP2 (Fig. 10*E*), which is capable of inhibiting MoSrcF and MoSrcFv *in vitro* (Fig. 10*D*). These observations suggest that MoSrcF and MoSrcFv are functionally active in *M. ovata*. Although choanoflagellates are generally unicellular, some can spontaneously form organized colonies (39) or create junction-like structures between cells (28). These observations demonstrate that the choanoflagellates can perform cell–cell interactions under certain conditions, probably mediated by cadherin-like and C-type lectin-like molecules (31, 32). Therefore, it is possible that *M. ovata* Src is involved in the regulation of such cell–cell interactions.

At the transition from unicellular to multicellular organisms, there must have been functional developments in various key molecules, including signaling molecules, adhesion molecules, and transcription factors, most of which are precisely regulated in multicellular animals. Therefore, systematic comparative analyses of the regulatory features of signaling molecules between the unicellular choanoflagellates and the multicellular sponge would provide previously undescribed insights into the molecular events that occurred at the critical transition of animal history.

Materials and Methods

cDNA Cloning. Full-length cDNAs of EfSrc and EfCsk were obtained as described in ref. 27. Partial cDNAs for MoSrc and MoCsk were amplified from poly(A)+ RNA extracted from *M. ovata* by high-fidelity PCR with degenerate primers. Full-length cDNAs then were obtained by 3′ and 5′ RACE. cDNAs were cloned into pT7Blue (Novagen, San Diego, CA) and sequenced by using a BigDye sequencing kit (ABI). The cDNAs and their mutant constructs were cloned into pCMV-Tag1 (for C-terminal myc tagging of Src; Stratagene, La Jolla, CA) or pMH (for C-terminal HA tagging of Csk; Roche) for expression in mammalian cells and into pESC-TRP (for C-terminal FLAG tagging of Src; Stratagene) or pESC-LEU (for C-terminal myc tagging of Csk) for expression in *Saccharomyces cerevisiae*. In addition, untagged vectors were generated to confirm that the tag sequences had no effect on enzyme function. The expression vectors for wild-type human Fyn (pMEM-hFyn) and its active form (pMEM-hFyn-F) were provided by T. Yamamoto (University of Tokyo, Tokyo, Japan). Mouse retroviral DNA constructs of pCX4pur-c-Src, and its activated mutants pCX4pur-c-Src(Y527F), pCX4bsr-v-Src, and pCX4, were prepared as described in ref. 36. MoSrc cDNAs were subcloned into pCX4bleo, and MoCsk cDNA was subcloned into pCX4neo.

Cell Culture. Csk-deficient cells and 293T cells were maintained in DMEM supplemented with 10% FBS. Transformation of *S. cerevisiae* was carried out according to the Stratagene protocol. After induction of protein expression in galactose medium, cells were resuspended in lysis buffer [20 mM Tris·HCl, pH 8.0/1 mM EDTA/0.15 M NaCl/1% Nonidet P-40/2% octyl-D-glucoside/1 mM Na_3VO_4/10 mM NaF/5% glycerol/a protease inhibitor mixture (Nakarai, Kyoto, Japan)]. The lysed sample was subjected to Western blotting as described in ref. 38. Immortalized rat embryonic fibroblast cells were prepared by introducing the SV40 large T-antigen (36). These cells were maintained in DMEM supplemented with 10% FBS. Stable cell lines expressing c-Src,

c-Src(Y527F), v-Src, MoSrc, or MoCsk were established by infecting cells with the respective retroviral expression vectors, followed by treatment with appropriate selective drugs as described in ref. 36. *M. ovata* (ATCC50635) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in the presence of bacteria at 20°C in ATCC medium 802. For the MoSrc inhibition assay, PP2 (Calbiochem, San Diego, CA) or the control compound PP3 were added to *M. ovata* or to the cultures of 293T cells expressing MoSrc.

Cell Transfection, Immunoprecipitation, and Western Blotting. For transient expression in 293T cells, cDNA (0.5 μg per well in six-well culture dishes) was transfected by using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), and cells were lysed in 250 μl of lysis buffer. Cleared lysates (10 μg of protein per lane) were separated on an SDS/PAGE gel and Western blotted as described in ref. 38. Immunoprecipitation assays were carried out as described in ref. 38. The following peptides were used for the anti-pY421 competition assay: RLEDFFVNTDSYTEAN and RLEDFFVNTD-SpYTEAN. For *in vitro* kinase assays, the immunoprecipitated Src was incubated in a reaction mixture (25 μl) consisting of 50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 50 μM ATP, 0.5 mM Na_3VO_4, 1 mM 2-mercaptoethanol, and 1 μg of GST-cortactin. After incubation for 10 min at 30°C, cortactin phosphorylation was analyzed by Western blotting with anti-pY421 antibody. Signal intensities were quantified by using the Image J program (National Institutes of Health). GST-cortactin was expressed and purified from Sf9 cells by using the Bac-to-Bac system (Invitrogen). Antisera to MoSrcF and MoSrcFv were raised in rabbits against GST fusion proteins containing their N-terminal unique regions (amino acids 1–46 for MoSrcF; amino acids 1–44 for MoSrcFv), and the antibodies were affinity purified on Affi-Gel (Bio-Rad, Hercules, CA) columns coupled with maltose binding protein-fusion proteins containing the same antigens. Western blotting was performed by using the following antibodies: anti-phosphotyrosine (4G10; Upstate Biotechnology, Charlottesville, VA); anti-Myc (PL14; MBL, Woburn, MA); anti-Csk (40); anti-Src (Ab-1; Oncogene, San Diego, CA); anti-Src pY418 (Biosource; Camarillo, CA); anti-Src pY529 (Biosource); anti-HA (Zymed, Temecula, CA); anti-FLAG (Sigma, St. Louis, MO); anti-cortactin (4F11; Upstate Biotechnology); anti-cortactin pY421 (Biosource); and anti-Fyn (Fyn3; Santa Cruz Biotechnology, Santa Cruz, CA).

Soft-Agar Colony Formation Assay. Transforming activity of the cells was examined by using a soft-agar colony formation assay. Cells were plated at 4×10^4 in 60-mm culture dishes in 3 ml of DMEM containing 0.36% agar on a layer of 5 ml of the same medium containing 0.7% agar. Five days after plating, cell colonies were stained with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (Sigma) and photographed.

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