

Review

Regulation of the Src Family Kinases by Csk

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Abstract

The non-receptor tyrosine kinase Csk serves as an indispensable negative regulator of the Src family tyrosine kinases (SFKs) by specifically phosphorylating the negative regulatory site of SFKs, thereby suppressing their oncogenic potential. Csk is primarily regulated through its SH2 domain, which is required for membrane translocation of Csk via binding to scaffold proteins such as Cbp/PAG1. The binding of scaffolds to the SH2 domain can also upregulate Csk kinase activity. These regulatory features have been elucidated by analyses of Csk structure at the atomic levels. Although Csk itself may not be mutated in human cancers, perturbation of the regulatory system consisting of Csk, Cbp/PAG1, or other scaffolds, and certain tyrosine phosphatases may explain the upregulation of SFKs frequently observed in human cancers. This review focuses on the molecular bases for the function, structure, and regulation of Csk as a unique regulatory tyrosine kinase for SFKs.

Key words: Csk, Src family, tyrosine kinases

The Src family tyrosine kinases

Tyrosine kinases, which exist only in animals, play important roles in controlling animal-specific cellular functions such as rapid cell-cell communication via the plasma membrane [1, 2]. The most critical feature of tyrosine kinases is the strict regulation of their functions. Due to these enzyme's importance in controlling cellular functions, dysregulation of tyrosine kinases can play a causative role in various diseases, especially cancers. Tyrosine kinases are classified into two major subgroups: receptor and non-receptor. The receptor tyrosine kinases are activated and auto-phosphorylated upon directly receiving extra-cellular cues, creating an intracellular platform for specific adaptors and effectors that activate downstream signaling pathways. The cytoplasmic non-receptor tyrosine kinases are also activated in response to extra-cellular cues via physical and functional interactions with particular transmembrane receptors, and subsequently activate specific downstream pathways to control cell fate.

The Src family tyrosine kinases (SFKs) are the major non-receptor tyrosine kinases expressed in multiple types of animal cells [3]. The SFKs include eight members, c-Src, c-Yes, Fyn, c-Fgr, Lyn, Hck, Lck, Blk. They are activated in concert with the activation of transmembrane receptors, such as EGF receptor, T cell receptor, and integrins. The SFKs regulate a wide range of cellular events, including cell growth, division, differentiation, survival, and programmed death, as well as specialized functions such as immune responses, cell adhesion, cell movement, and endocytosis [4, 5]. Several of the SFKs, c-Src, c-Yes, and c-Fgr, were originally discovered as prototypes of avian retroviral oncogene products (v-Src, v-Yes and v-Fgr) [6-11]. Dysregulation of SFKs has been implicated in the etiology of various human diseases such as cancers, although somatic mutation in the SFK genes has not been detected [12, 13]. These observations underscore the important roles played by SFKs. However, the molecular mechanisms underlying the

cells, SFKs are mostly phosphorylated at Tyr-527, and adopt the inactive conformation, which is stabilized by two intramolecular interactions: 1) binding of phosphorylated Tyr-527 to its own SH2 domain, and 2) binding of the SH2-kinase linker to the SH3 domain. These intramolecular interactions affect the configuration of the catalytic pocket. In particular, the hydrogen bond between Glu-310 in the α C-helix and Lys-295, required for Mg-ATP binding, is disrupted in the inactive conformation. The dephosphorylation of Tyr-527 releases the 'lock' by the SH2 domain and causes dramatic conformational change in the kinase domain, resulting in formation of a hydrogen bond between Glu-310 and Lys-295 in the catalytic domain (Fig 2A). This active enzyme can now catalyze the intermolecular auto-phosphorylation of Tyr-416 in the activation loop. This auto-phosphorylation locks the catalytic domain into the active conformation and facilitates access of substrates to the active site [22].

The regulation of SFKs by intramolecular interactions suggests that SFKs can also be regulated by interaction with molecules that compete with the SFK domains. Indeed, SFKs bind to various tyrosine-phosphorylated proteins by recognizing specific phosphopeptide sequences via the SH2 domain [23]. For example, c-Src binds to the phosphorylated forms of Cas, FAK, and paxillin, as well as growth factor receptors such as EGFR, CSF-1 and PDGF, resulting in activation of c-Src [5, 24-26]. Activated c-Src can further phosphorylate these interacting proteins to create new binding sites for other adaptors and effectors, which in turn allows amplification of signals. In addition, the c-Src SH3 domain binds to various signaling proteins that contain proline-rich motifs [27], such as Shc, PI3K, Cas, Tks5, and Arhgef5 [28-30]. Interactions with these proteins disrupt the stabilized inactive conformations of SFKs and promote the phosphorylation of these proteins by SFKs.

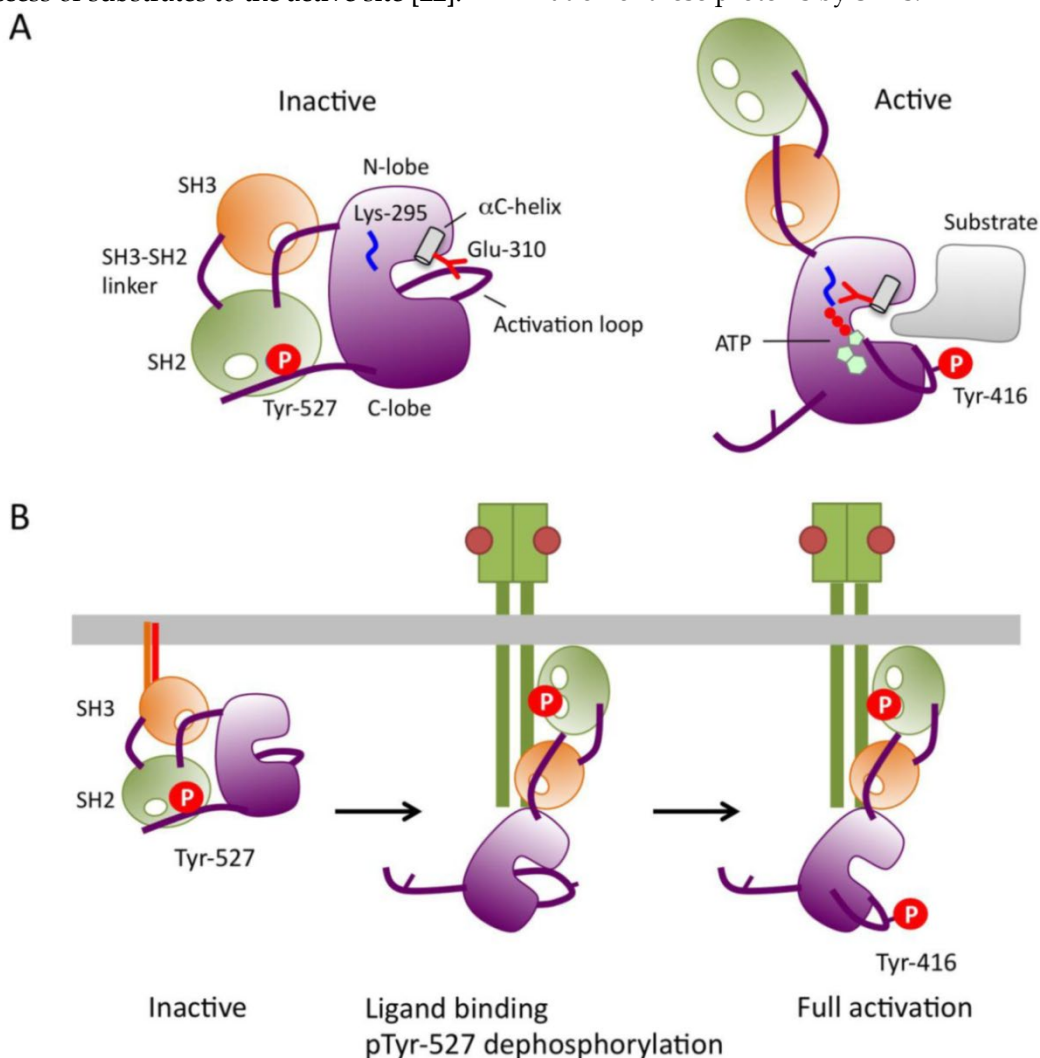


Fig.2. Mechanism of SFK activation. (A) Schematic models of inactive and active forms of c-Src (cited from [21]). (B) A predicted mechanism of SFK activation.

Although the molecular mechanisms of SFK activation may vary depending upon cell types and extracellular cues, it is now believed that in general, full activation of SFK is achieved in the following order (Fig.2B): 1) the activated receptors, adaptors, or effectors interact with the SH2/3 domains of inactive SFKs to open up the closed conformation, and harbor SFKs at appropriate intracellular locations such as focal adhesions; and 2) tyrosine phosphatases dephosphorylate the exposed pTyr-527 to stabilize the active conformation; and 3) the activated SFKs undergo intermolecular auto-phosphorylation on Tyr-416 to lock the catalytic pocket into the fully active conformation. The fully activated SFKs then phosphorylate substrate proteins, such as FAK and Cas, many of which can also create binding sites for SFKs; this initiates the positive-feedback loop of SFK activation. When the cell response is terminated, activated SFKs are rapidly degraded via the ubiquitin-proteasome pathway [31] or inactivated by phosphorylation at Tyr-527. Thus, in order to guarantee rapid cell response to extracellular cues, most of SFKs in the resting state should be inactivated by phosphorylation at Tyr-527. Furthermore, this highly secure regulatory system is required in order to strictly control potentially dangerous SFKs that possess inherent oncogenic activity. However, the tyrosine kinase responsible for Tyr-527 phosphorylation was not identified until we discovered Csk (C-terminal Src Kinase). In the next chapter, I will introduce Csk as a genuine suppressor of 'dangerous' SFKs.

Discovery of Csk

In the late 80's, my research focused on the roles of c-Src in the terminal differentiation of neurons. c-Src⁺ (neuronal c-Src or n-Src), an alternative splicing variant having 6 amino acid insertion in the SH3 domain, is abundantly expressed specifically in post-mitotic neurons [32, 33]. To elucidate the function and regulation of c-Src⁺, I first attempted to purify the SFK-related tyrosine kinases from neonatal rat brains, a rich source of developing neurons, by means of column chromatography. I successfully isolated c-Src⁺ and a new tyrosine kinase that was co-purified with c-Src⁺ [34] (Fig.3A). Because I realized that the new kinase might be involved specifically in the regulation of c-Src⁺, I challenged it with the purified c-Src⁺ protein to determine whether it was the missing Tyr-527 kinase. At least *in vitro*, the answer was "Yes" [35]. Subsequent biochemical analysis confirmed that the kinase (named Csk, from C-terminal Src kinase) [36] specifically phosphorylates Tyr-527 and can inhibit the activity of c-Src (Fig.3B). Next, we cloned rat Csk cDNA and confirmed its specificity for c-Src in a yeast

model system [37]. Furthermore, we and another group showed that Csk phosphorylates the conserved negative regulatory sites in other SFKs [36, 38]. Sabe *et al.* showed that when Csk is overexpressed, it inhibits transformation of fibroblasts by the combined actions of c-Src and the oncoprotein v-Crk [39, 40]. This observation suggests that Csk has the potential to serve as a repressor of c-Src *in vivo* as well as *in vitro*.

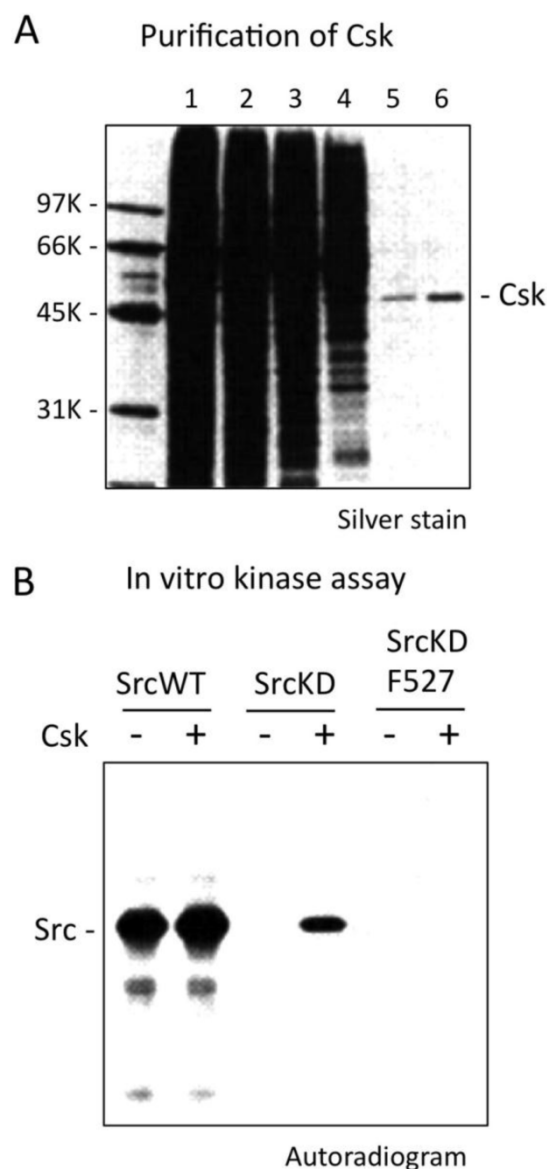


Fig.3. Discovery of Csk. (A) Sample of each purification step of Csk was analysed by SDS-PAGE and staining with silver. Lane 1, Nonidet P-40 extract; lane 2, DEAE-cellulose column chromatography; lane 3, poly(Glu, Tyr) Sepharose CL-4B; lane 4, Mono Q; lane 5, Sephacryl S200HR; lane 6, Mono S. (B) Phosphorylation of Src by Csk was determined by incubating immunopurified SrcWT (lanes 1 and 2), SrcKD (lanes 3 and 4) and SrcKDF527 (lanes 5 and 6) with or without Csk. The reaction products were analyzed by SDS-PAGE and autoradiography.

Function of Csk

The function of Csk as a negative regulator of SFKs *in vivo* was demonstrated by the phenotypes of mice lacking Csk [41, 42] (Fig.4A). Homozygous mutant embryos die at neurulation stages with defects in neural tube closure. Mutant embryonal tissues and established Csk-deficient cell lines exhibit increased activity of all SFKs tested, resulting in the accumulation of tyrosine-phosphorylated proteins. These findings strongly suggest that Csk is required to repress SFKs *in vivo*. Conditional ablation of Csk in specific tissues also causes severe defects associated with constitutive activation of SFKs. Loss of Csk induces dysfunction in acute inflammatory responses [43] and T-cell development [44], hyperplasia of the epidermis [45] and defects in cell adhesion and migration [46]. Even in invertebrates, such as *Drosophila* and *C. elegans*, the loss of Csk leads to constitutive activation of SFKs, causing hyperproliferation and defective cytoskeletal function, respectively [47, 48]. These findings indicate that Csk is an indispensable regulator of SFKs (Fig.4B).

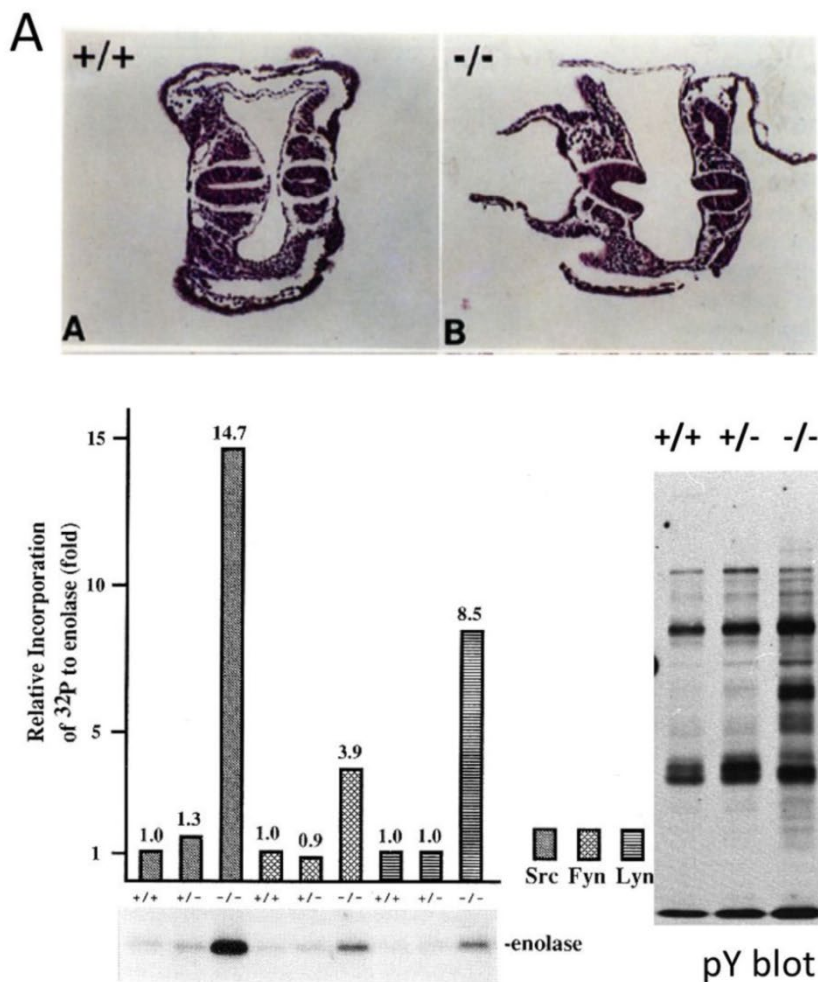


Fig.4. Function of Csk. (A: upper) Histological appearances of E8.0 embryos. Transverse sections through the trunk region of wild-type (+/+) and Csk KO (-/-) embryos, respectively. The neural tube began to close in the wild-type embryo, but not in the homozygous mutant embryos. (A: lower left) Activation of SFKs in Csk KO embryos. Src, Fyn and Lyn were immunopurified from the indicated genotypes of embryos and incubated with acid-treated enolase and [γ -³²P]ATP, followed by SDS-PAGE. The incorporation of ³²P to enolase was quantitated. (A: lower right) Tyrosine-phosphorylated proteins in the indicated genotypes of embryos were analyzed by Western blotting with anti-phosphotyrosine antibody. (B) Csk can phosphorylate the C-terminal regulatory sites of all the members of SFKs to repress their activities.

To further verify the functional connections between Csk and SFKs, we investigated homologs of SFKs and Csk in two primitive animals; a unicellular

choanoflagellate *M. ovate* and a fresh-water sponge *E. fluviatilis* (Fig.5).

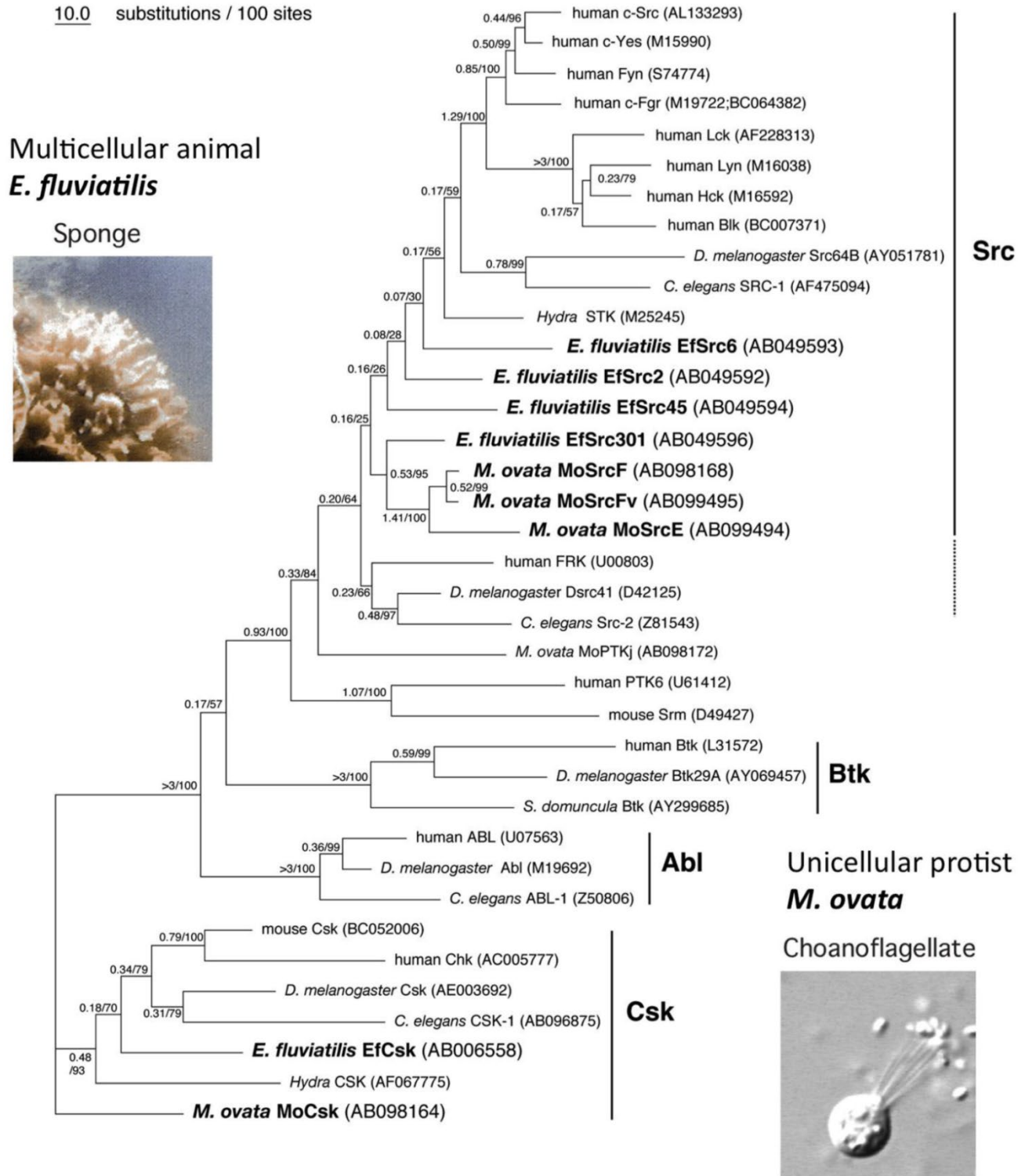


Fig.5. Evolution of SFKs and Csk. Phylogenetic tree of Src, Csk, and related genes by using the maximum likelihood method. The reliability index (1) and bootstrap probability (2) for each branch are indicated before and after the slash, respectively.

Sequence alignment of invertebrate and vertebrate genes reveal that Csk is highly conserved across the animal kingdom, from the unicellular choanoflagellate to humans, as are the SFKs [49], confirming the intimate relationship between Csk and SFKs. We also found that SFKs form a gene family even in unicellular choanoflagellates, whereas Csk is present as a single gene in all animal phyla: this is consistent with the idea that Csk is the sole conductor of SFKs (Fig.4B). Furthermore, we showed that the SFKs in the unicellular choanoflagellates are more resistant to Csk-mediated inactivation than those of multicellular animals, suggesting that strict regulation of SFKs has been established during the evolution of the multicellular animals [49]. This may mean that the strict 'ON-OFF' regulation of SFKs is prerequisite for the evolution of multicellular organization. The loss of this regulatory system, e.g., upon expression of v-Src, may lead to dysregulation of normal cellular interactions such as that observed in cancers.

A tyrosine kinase homologous to Csk, formerly named Matk (Ctk, Hyl, Chk, or Lsk), has been identified in mammalian hematopoietic cells [50-55]. The results of *in vitro* analyses suggest that, like Csk, Matk can target SFKs to repress their activity. However, analyses of Matk-deficient mice revealed that loss of Matk did not result in any overt phenotype or affect the activity of SFKs in cells, suggesting that it might not have the same function as Csk [56]. Thus, it is likely that Csk is the major kinase involved in the regulation of SFKs *in vivo*, and Matk may play specific roles in hematopoietic cells.

As a protein tyrosine kinase, Csk is exceptional in that it exhibits high substrate specificity toward the C-terminal regulatory tyrosine (equivalent to Tyr-527) of SFKs. The surrounding sequence of the SFK regulatory site (QYQ) is highly conserved among SFKs, but biochemical and structural studies reveal that another region (aa 504-525) located distantly from Tyr-527 is rather crucial for specific recognition by Csk [57-59] (Fig.1). In addition to SFKs, several signaling proteins have been reported to serve as substrates of Csk. Those include paxillin [60], P2X₃ receptor [61], c-Jun [62] and Lats [63]. However, the physiological relevance of the phosphorylation of these proteins remains unclear.

Although the kinase Csk has been identified as a negative regulator of SFKs, the phosphatases that activate SFKs by acting on Tyr-527 have not yet been identified with certainty. So far, several candidate phosphatases have been reported [64]: those include PTP α [65], PTP ϵ [66], PTP λ [67], CD45 [68] and PTP1B [69], SHP-1 [70], and SHP-2 [71]. However, the substrate specificity of these tyrosine phosphatases is not

as strict as that of Csk; most of them can dephosphorylate both Tyr-527 and Tyr-416. These findings imply that specific phosphatase may not be involved in the direct activation of SFKs, and the dephosphorylation of Tyr-527 may be a secondary event that occurs after the ligand-mediated conformational changes earlier in the process of SFK activation.

Structure of Csk

Csk is a non-receptor tyrosine kinase with a molecular mass of 50 kDa. It contains the SH3 and SH2 domains in its N-terminus and a kinase domain in its C-terminus [37]. This arrangement of functional domains within the primary structure is similar to that of SFKs, but Csk lacks the N-terminal fatty acylation sites, the auto-phosphorylation site in the activation loop, and the C-terminal negative regulatory sites, all of which are conserved among SFK proteins and critical for their proper regulation (Fig.6A). The absence of auto-phosphorylation in the activation loop is a distinguishing feature of Csk. The crystal structure of full-length Csk reveals significantly different dispositions of the functional domains relative to those of SFKs [72], indicating that Csk is regulated differently than SFKs (Fig.6B). The most striking feature of the Csk structure is that, unlike the situation in SFKs, the binding pockets of the SH3 and SH2 domains are oriented outward, enabling intermolecular interactions with other molecules. The Csk crystal we analyzed consists of six molecules classified as either active or inactive states according to the coordination states of catalytic residues (specifically, the presence or absence of the salt bridge between Glu236 and Lys222) (Fig.7). In active molecules, the SH2-kinase and SH2-SH3 linkers are tightly bound to the N-terminal lobe of the kinase domain in order to stabilize the active conformation, and there is a direct linkage between the SH2 and the kinase domains. In inactive molecules, the SH2 domains are rotated in a manner that disrupts the linkage to the kinase domain. These observations suggest that the active and inactive conformations of Csk exist in a dynamic equilibrium, and that overall activity is regulated through binding to the SH2 domain of some other factors: such binding would lock the SH2 and kinase domains into the coupled state [72, 73].

The extensive mutagenesis study located the substrate-docking sites on the surface of Csk, providing a structural basis for the high substrate specificity of Csk [58]. The crystal structure of a complex between the kinase domains of Csk and c-Src further revealed that interactions between these kinases position the C-terminal tail of c-Src at the edge

of the active site of Csk [59]. Based on this finding, those authors suggested that Csk cannot phosphorylate substrates that lack this docking mechanism, because the conventional substrate binding site is destabilized in Csk by the lack of auto-phosphorylation

in the activation loop. Taken together, it is likely that the distinguishing features of Csk structure, *i.e.*, the lack of auto-phosphorylation and the specific orientations of the domains, may determine its exceptionally high substrate specificity.

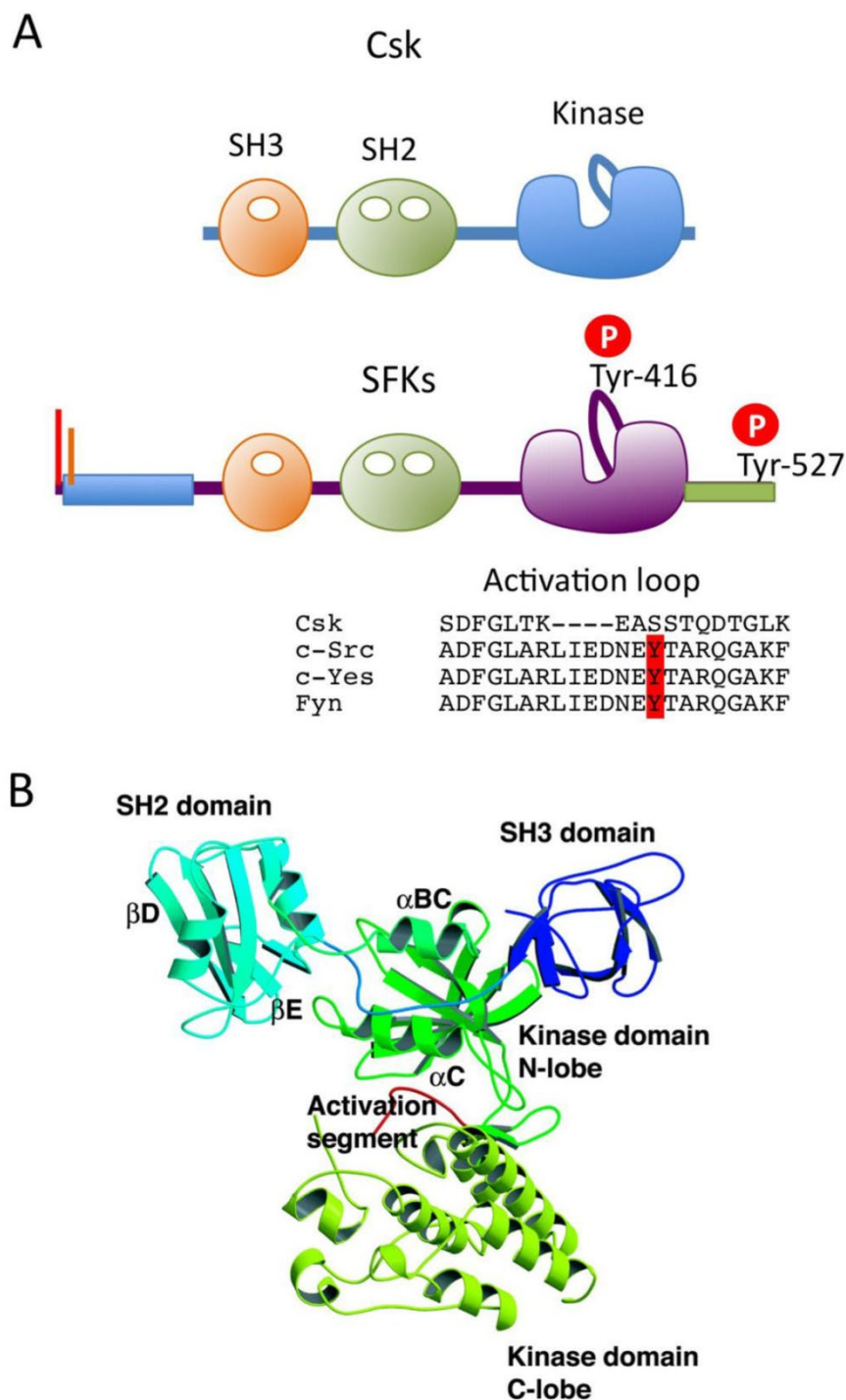


Fig.6. Structure of Csk. (A) Domain organizations of Csk and SFKs. An amino acid alignment of the activation loop of Csk and SFKs is also shown. (B) Ribon diagram of a representative Csk structure (active molecule) in the crystal.

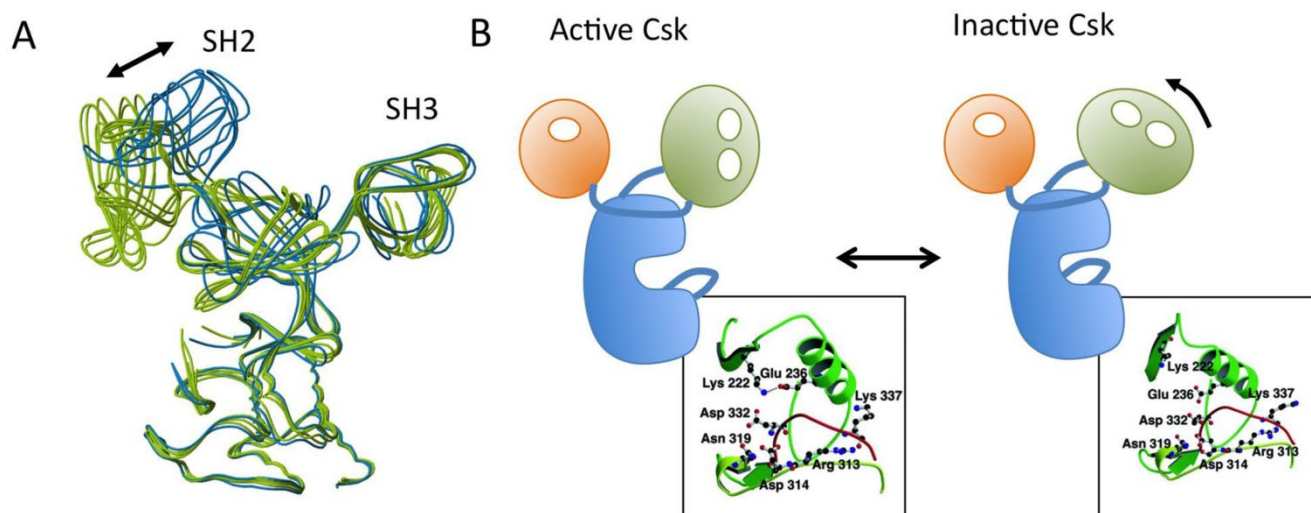


Fig.7. Regulation of Csk. (A) Superimposed model of the six Csk molecules in an asymmetric unit. Active molecules are shown in green and inactive molecules are in blue. (B) Schematic models of active and inactive Csk structures. (B: boxes) Arrangements of the catalytically important residues in active and inactive Csk. The salt bridge between Lys-222 and Glu-236 is indicated by dotted lines.

Regulation of Csk

Because Csk lacks a transmembrane domain and fatty acyl modifications, it is predominantly present in cytosol, whereas its substrate SFKs are anchored to the membrane via their N-terminal myristate and palmitate moieties. Therefore, the translocation of Csk to the membrane, where SFKs are activated, is thought to be a critical step of Csk regulation. So far, several scaffolding proteins, *e.g.*, caveolin-1, paxillin, Dab2, VE-cadherin, IGF-1R, IR, LIME, and SIT1, have been identified as membrane anchors of Csk [74-81]. We also searched for intrinsic phosphoproteins that might be involved in the regulation of Csk by binding to the SH2 domain, and successfully isolated a transmembrane adaptor-like molecule Cbp/PAG1 (Csk binding protein/phosphoprotein associated with glycosphingolipid-enriched membrane) from the cholesterol-enriched membrane microdomains "lipid rafts" [82, 83]. Cbp has a single transmembrane domain at its N-terminus and two palmitoyl modification sites just C-terminal to the transmembrane domain, through which Cbp is exclusively localized to lipid rafts. Cbp also contains nine tyrosine residues that can be phosphorylated, four of which are in the typical Src SH2 binding motifs (YEEI/V), and one of which is the Csk SH2 binding site (YSSV). Because Cbp is co-localized with SFKs in lipid rafts, it is a readily available substrate of SFKs, and its five SH2 binding motifs are efficiently phosphorylated. Single molecules of phosphorylated Cbp can simultaneously bind Csk and SFKs, thereby bringing them into close proximity and enabling efficient inactivation of the SFKs by Csk [14, 84, 85] (Fig.8). This negative feed-

back loop is crucial in preventing tumorigenesis and controlling the cell signaling evoked by activation of growth factor receptors. We also found that Cbp is downregulated by activation of not only SFKs but also other oncogenes such as Ras [84]. It is also downregulated in various cancer cells, potentially through epigenetic silencing [86]; this observation may explain the frequent upregulation of SFK function in cancer cells that lack somatic mutations in SFKs. Other Csk-binding proteins, such as paxillin [75] and caveolin-1 [74], are also phosphorylated by SFKs and participate in the negative regulation of SFKs by recruiting Csk to the sites of SFK activation [87].

The binding of scaffolds to the SH2 domain of Csk can also activate the enzyme activity of Csk. The occupation of the SH2 domain of Csk by phosphorylated Cbp affects conformation of the catalytic domain, thereby increasing Csk activity toward SFKs [73, 88]. Thus, it is likely that the scaffold proteins positively regulate Csk functions not only by recruiting Csk to the membrane but also by directly activating it. On the other hand, Cloutier and Veillette found that Csk can associate with the protein tyrosine phosphatase PEP/LYP expressed in hemopoietic cells [89, 90]. Their results suggest that the association of Csk with PEP/LYP constitutes a more efficient means of inhibiting signal transduction by SFKs *in vivo*. However, a more recent report presented results that call into question the postulated function of LYP-Csk interactions [91, 92]. Another regulatory mechanism is suggested by observations that the activity of Csk can be regulated by the oxidation state of the disulfide bond in the SH2 domain, implying that Csk could be

regulated by the redox state within the cells [93]. Furthermore, Csk is phosphorylated by PKA at Ser-364, resulting in an increase in kinase activity [94]. However, the physiological relevance of this phosphorylation event has not yet been addressed. Csk is highly expressed in the developing nervous system and lymphoid cells [95], but the mechanisms underlying the regulation of Csk at the expression levels remains to be elucidated

Csk in diseases

Accumulating evidence suggests that Csk may play a role in tumor suppression by inhibiting the oncogenic activity of SFKs. Indeed, overexpression of Csk can inhibit tumor growth of human colon cancer cells [84]. Heterozygous *csk*^{+/-} mutant mice exhibit higher sensitivity to chemically induced skin carcinogenesis than wild-type mice (Yagi et al., unpublished observation) (Fig.9). However, there is no convincing evidence that supports the direct involvement of Csk in human cancer. Although Csk is downregulated in some cancers [96], it is likely that Csk is expressed at normal levels in many types of cancer cells [97], and, like the *SRC* gene [12], there have been no reports of

somatic mutation in the *csk* gene. By contrast, as mentioned above, the expression of Cbp/PAG1 is downregulated in multiple types of cancer cells [84], potentially via an epigenetic mechanism [86]. The downregulation of Cbp/PAG1 may interfere with the translocation of Csk to the membrane, suppressing the inactivation of SFKs. This mechanism may explain the upregulation of SFKs in some cancer cells. Therefore, further analyses of Cbp/PAG1-mediated regulatory system should reveal new opportunities for therapeutic intervention in cancer.

Perspectives

The function of Csk as a regulator of SFKs has been almost completely established, but the question of whether or not Csk has additional functions still remains to be addressed. The greater question of why Csk and SFKs are not mutated in human diseases should also be addressed. More comprehensive search for these mutations in human diseases would give a new clue for understanding the biological significance of the intimate relationship between SFKs and Csk.

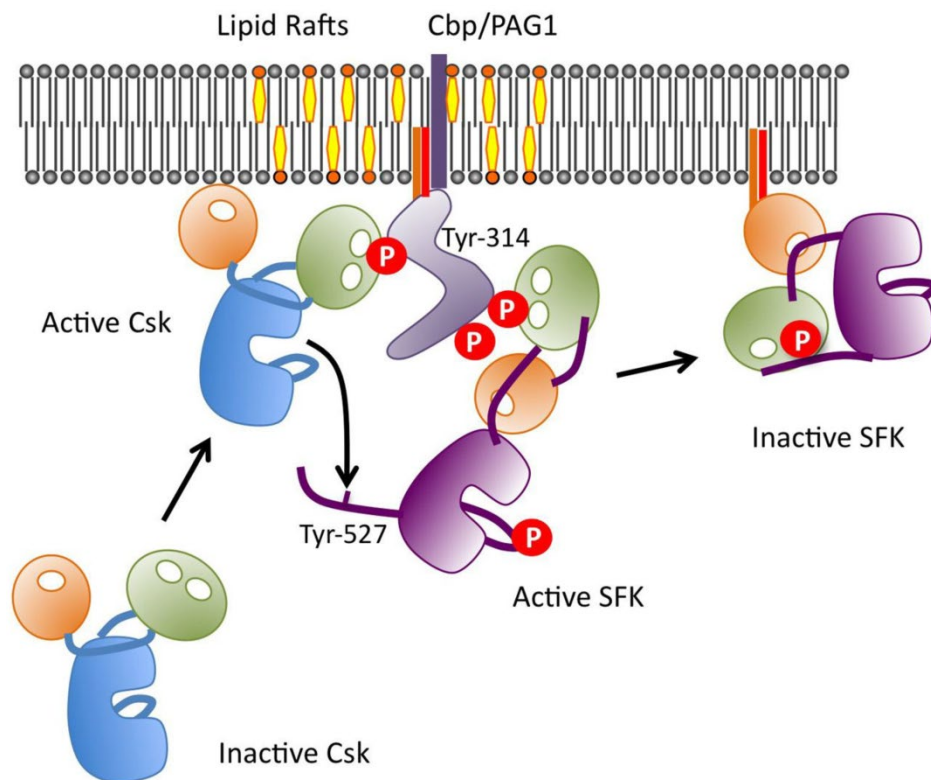


Fig.8. Regulation of Csk via Cbp in lipid rafts. A schematic model of the roles of Cbp/Csk and lipid rafts in regulating the function of SFKs. When Cbp is phosphorylated by active SFKs, Csk is recruited to lipid rafts via binding to Cbp at pY314 and phosphorylates Y527 to inactivate the catalytic activity of SFKs. The inactivated SFKs then relocate to non-raft compartments.

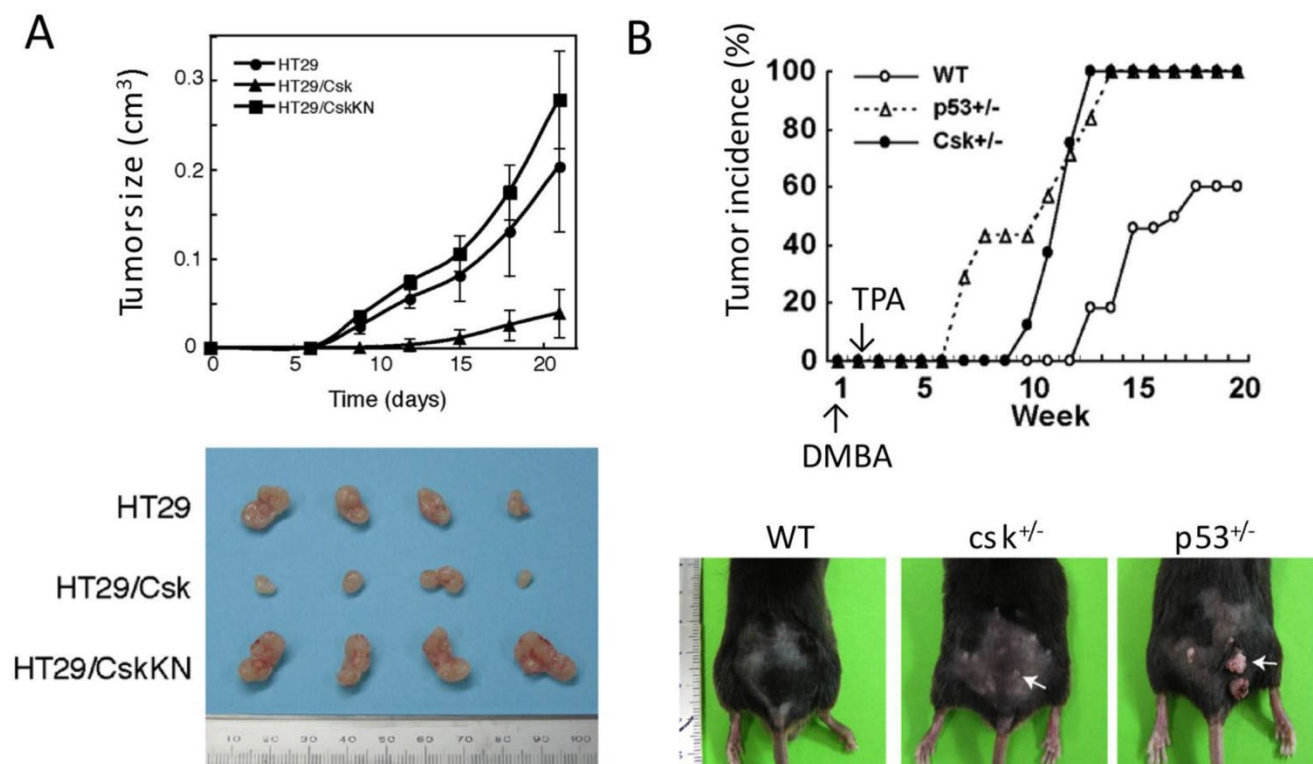


Fig.9. Function of Csk as a tumor suppressor. (A) Effect of Csk overexpression on tumor growth. Human colon cancer cell line HT29, HT29 expressing wild-type Csk (HT29/Csk) and HT29 expressing kinase-negative Csk (HT29/CskKN) were injected s.c. into nude mice. Tumor volume (cm³) obtained from four mice is plotted versus days after inoculation. Excised tumors are shown in lower panel. (B) Tumor incidence in wild-type and heterozygous for Csk (*csk*^{+/-}) and p53 (*p53*^{+/-}) after the sequential treatment of skin with dimethyl benzanthracene (DMBA) and phorbol myristate acetate (TPA).

Competing Interests

The author has declared that no competing interest exists.

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