Functions of the Activation Loop in Csk Protein Tyrosine Kinase†

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Running title: Activation Loop of Csk Protein Tyrosine Kinase

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Footnote:

1. Abbreviations used: Chk, Csk-homologous kinase; CSAL, Csk with a Src activation loop; Csk, C-terminal Src kinase; Δ339, Csk mutant in which residue 339 is deleted; GAL, Csk with all activation loop residues replaced by Gly; GCSP, GST fused to Csk substrate peptide; GST, glutathione S-transferase; kdSrc, kinase-defective Src; PTK, protein tyrosine kinase; RCM-L, carboxymethylated-maleylated, reduced lysozyme; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TC-Csk, thrombin cleavable Csk.
Abstract

Autophosphorylation in the activation loop is a common mechanism regulating the activities of protein tyrosine kinases (PTKs). PTKs in the Csk family, Csk and Chk, are rare exceptions for lacking Tyr residues in this loop. We probed the function of this loop in Csk by extensive site-specific mutagenesis and kinetic studies using physiological and artificial substrates. These studies led to several surprising conclusions. First, specific residues in Csk activation loop had little discernable functions in phosphorylation of its physiological substrate Src, as Ala scanning and loop replacement mutations decreased Csk activity toward Src less than 40%. Second, some activation loop mutants, such as a single residue deletion or replacing all residues with Gly, exhibited 1-2% of wt activity toward artificial substrates, but significantly higher activity toward Src. Third, introduction of a thrombin cleavage site to the activation also resulted in loss of 98% of wt activity for polyE_4Y and loss of 95% of wt activity toward Src, but digestion with thrombin to cut the activation loop, resulted in full recovery of wt activity toward both substrates. This suggested that the catalytic machinery is fully functional without the activation loop, implying an inhibitory role by the activation loop as a regulatory structure. Fourth, Arg313, although universally conserved in protein kinases, and essential for the activity of other PTKs so far tested, is not important for Csk activity. These findings provide new perspectives for understanding autophosphorylation as a regulatory mechanism, and imply key differences in Csk recognition of artificial and physiological substrates.
Protein tyrosine phosphorylation plays crucial roles in regulating cell proliferation and differentiation, the cell cycle, and transmission of various signals from the extracellular environment to a cell (1). This post-translational modification is catalyzed by protein tyrosine kinases (PTKs), a large class of enzymes that transfer the $\gamma$-phosphate of ATP to specific tyrosine residues in their protein substrates. Due to their critical roles in cellular regulation, the activities of PTKs in the cells are tightly controlled. Autophosphorylation is a key mechanism regulating the activity of most PTKs (2, 3). It occurs on one or multiple Tyr residues on a loop located in the kinase domain, called the autophosphorylation loop. Since autophosphorylation activates the kinases, this loop is also called the activation loop.

The catalytic domain of a PTK consists of an ATP-binding lobe, a peptide binding lobe, and a cleft between the two lobes (4). The cleft is the active site and contains two loops: the activation loop and the catalytic loop. The activation loop controls kinase activities by assuming different phosphorylation dependent conformations (3, 5). In the unphosphorylated form, the activation loop blocks the access to the active site by interactions with residues from the catalytic loop and other substructures within the ATP- and peptide-binding lobes. In this conformation the autophosphorylatable Tyr forms a hydrogen bond with a catalytically essential Asp residue located in the catalytic loop (4, 6, 7). Autophosphorylation results in large conformational changes in the activation loop that moves away from the active site, making the active site accessible (8, 9). The active conformation is stabilized by interactions of the phospho-Tyr in the activation loop with two Arg residues, one from the catalytic loop and the other from the activation loop (8, 9).
In light of the virtual ubiquity of autophosphorylation as a key regulatory mechanism, it is intriguing that PTKs in the Csk family, C-terminal Src kinase (Csk) and Csk homologous kinase (Chk), do not contain a Tyr residue in the activation loop (10, 11). Csk and Chk are soluble PTKs that phosphorylate PTKs of the Src family on a C-terminal Tyr and negatively regulate their activities (12). The kinase domains of Csk (13, 14, Fig. 1A) and Chk share many structural features with other PTKs, such as the ATP-binding and peptide-binding lobes, and the active site cleft. Within the active site, Csk also contains the catalytic loop and the activation loop. Their catalytic loops share the consensus sequence of HRDLAARN with other PTKs. Like other PTKs, the Csk and Chk activation loops follows a Asp$^{332}$-Phe-Gly (DFG) motif on the amino end and connect to P+1 loop on the carboxyl end. But the Csk and Chk activation loops share little homology with other PTKs, or with each other. Since Csk and Chk do not contain a Tyr residue in the activation loop, they appear not regulated by autophosphorylation (15, 16). It is not clear if this loop is involved in some alternative regulatory mechanisms.

In this communication, we probed the role of the activation loop of Csk by site-specific mutagenesis and kinetic characterization. Several observations on this exceptional activation loop provide novel insights into the role of activation loops, substrate recognition and cooperation between the regulatory and catalytic machinery of the PTK structure.

**Experimental Procedures**

*Generation of Csk mutants.* (His)$_6$-tagged Csk was expressed using pET-Csk-H$_6$ (constructed by inserting human Csk coding sequence with a (His)$_6$-tag sequence at the 3'
end into the pET-23 a(+) in BL21 (DE3) *Escherichia coli*. The full-length Csk was extended by 6 His residues at the C-terminus to aid purification. Ala scanning mutants and other Csk point mutants were generated using QuikChange (Strategene) in the parental plasmid of pET-Csk-H$_6$. Loop replacement mutation (CSAL) was incorporated by polymerase chain reaction with primer extensions. All mutants were confirmed by DNA sequencing.

*Enzyme purification.* The wt and mutant Csk enzymes were purified to apparent or near homogeneity by Ni$^{2+}$-affinity chromatography as follows. Harvested cell paste was resuspended (culture volume versus resuspension volume = 50:1) in the binding buffer (100 mM Tris-Cl, and 5 mM imidazole, pH 8.0) and sonicated three times for 10 s each. The cell lysate was then cleared by centrifugation at 34,000 g for 10 min. The supernatant was applied to a Ni$^{2+}$-iminodiacetic acid-Agarose column (Sigma) equilibrated with the binding buffer. After the sample was loaded, the column was washed with 15 bed volumes of the wash buffer (100 mM Tris-Cl, and 60 mM imidazole, pH 8.0). The protein still bound to the column was eluted with elution buffer (100 mM Tris-Cl, and 200 mM imidazole, pH 8.0). The purified enzymes were desalted on a Sephadex G25 column equilibrated with the storage buffer (100 mM Tris-Cl, pH 8.0, and 0.1% β-mercaptopethanol). Glycerol was added to the purified fractions to 30% and the enzymes were stored at -20 °C. Protein concentrations were determined by Bradford assay and purity was assessed by SDS-PAGE with coomassie blue staining.
Kinase activity assay. For assaying PTK activities, we measured the phosphorylation of polyE₄Y and kdSrc using acid precipitation assay as previously described (17, 18). The kinase-defective Src (kdSrc) was avian Src that contained a Lys295Met mutation, which abolished its kinase activity (18, 19). When the $K_m$ and $k_{cat}$ were determined with regard to one substrate, kinase activity was determined at various concentrations of that substrate in the range of 20 to 200 $\mu$M for ATP, 20 to 200 $\mu$g ml$^{-1}$ for polyE₄Y, and 1 to 10 $\mu$M for kdSrc. The $k_{cat}$ and $K_m$ values were determined by Lineweaver-Burk plots with linear regression using the Microsoft Excel program. Carboxymethylated-maleylated, reduced lysozyme (RCM-L) and GST fused to the Csk substrate peptide (GCSP) were also used as substrates in certain assays. GCSP was generated by fusing the optimal Csk peptide sequence, EEIYFFF (20), to the carboxyl terminus of GST.

Thrombin digestion. Equal amounts (20 $\mu$g) of wt Csk, or the Csk mutant TC-Csk were incubated in the presence or absence of 0.8 $\mu$g thrombin (~ 2 NIH units) in the thrombin digestion buffer (50 mM Tris, pH 8.0) at 25 °C for 2 h. At the end of the digestion, aliquots of the protein samples were mixed with equal volumes of sample buffer and analyzed by SDS-PAGE. For enzymatic characterization of thrombin-digested TC-Csk, the digested enzymes were stored at 4 °C and used within the same day. Storage of the digestion mixture at 4 °C for up to one day did not result in detectable non-specific digestion or loss of kinase activity.

To determine if the two fragments produced by thrombin digestion were associated with each other, digested TC-Csk was purified by Ni$^{2+}$-affinity column as described
earlier. The purified proteins were compared to digested TC-Csk prior to this step of affinity purification to determine the co-purification of digested fragments.

Results

Ala scanning mutagenesis of the Csk activation loop. A comparison between active and inactive structures of Csk (14) indicated that many residues in the activation loop assume dramatically different conformations (Fig. 1B) in the two forms, suggesting the possibility that the conformation of the activation loop may correlate to the activation states of Csk. To evaluate the role of individual residues in this loop, all non-Ala residues were individually mutated to Ala. The kinase activities of mutants were determined using both polyE4Y and the kdSrc as substrates. Among the 10 mutants, most exhibited specific activity within 80% of the wt enzyme (Fig. 2). The most severe effect was observed in Glu338Ala, which retained approximately 40% and 60% of wt activity using polyE4Y and kdSrc as substrates, respectively. In contrast, mutation of Asp332 of the DFG motif in front of the activation loop to Ala abolished the kinase activity with either substrate. These results indicated that none of the mutated residues in the Csk activation loop played any essential roles in Csk catalysis or activation.

In other PTKs that are regulated by autophosphorylation, the pTyr in the activation loop is stabilized by interaction with two Arg residues. The first Arg, which is equivalent to Lys337 in Csk, is critical for the autophosphorylation-initiated activation of v-Fps (21), but our results demonstrated that Lys337 was not required for Csk activation. The second is an Arg from the catalytic loop, equivalent to Arg313, which is universally conserved. To determine if Arg313 is required for Csk activation, we mutated Arg313 to Ala.
Arg313Ala exhibited 87% and 108% of the wt activity toward polyE₄Y and kdSrc, respectively (Fig. 2). This result indicated that the side chain of Arg313 was not involved in essential catalytic or regulatory functions. This effect directly contrasts mutagenic studies of Src and v-FPS, in which mutations of the equivalent residues to Ala abolished the kinase activities (21, 22).

The Csk activation loop can be functionally replaced by an unrelated activation loop. Csk shares the highest overall amino acid sequence homology with PTKs of the Src family, but the two families share no significant homology in the activation loop. Src contains one Tyr residue in the activation loop, whose autophosphorylation activates Src kinase activity, and alters other regulatory properties (23-25). The Src activation loop is also four residues longer than the Csk counterpart. To determine if the Csk activation loop can be functionally replaced by the Src counterpart, a Csk mutant with a Src activation loop (CSAL) was generated. CSAL exhibited approximately 80% and 130% of the wt activity with polyE₄Y and kdSrc as substrates, respectively, indicating that the longer activation loop can be accommodated, and that none of the Csk specific residues in the activation loop was required for the kinase activity. The Ala scanning and the loop replacement results indicate that Csk is active without the help of activation loop side chains.

We then determined if CSAL, containing a Tyr residue in the activation loop, could autophosphorylate and if the phosphorylation of the Tyr residue could activate CSAL (Fig. 3). Incubation of the mutant enzyme with 0.1 mM ATP and 12 mM Mg²⁺ did not result in autophosphorylation higher than that of wt Csk. When incubated with Src in the presence of ATP and MgCl₂, CSAL, but not Csk, was readily phosphorylated in a
time-dependent manner. This result indicated that the amino acid sequence in the activation loop contained the necessary structural information to be phosphorylated by Src, but not by Csk. The phosphorylation of CSAL by Src is also consistent with the trans-mechanism for Src autophosphorylation (26). No effect on CSAL activity was detected when the phosphorylation level reached ~30% (data not shown).

Probing the conformational role of the Csk activation loop. The apparent lack of a functional role by the activation loop contradicted our expectations based on its central location and the conformational differences between active and inactive Csk. We further probed the role of the activation loop by additional more aggressive mutations (Table 1).

We determined if the overall length of the loop was important by deleting Ala339 (∆339). This mutant retained approximately 1.8% of the wt activity toward polyE₄Y, and 20% wt activity toward kdSrc. Since the Ala side chain was unlikely to be essential for catalysis or regulation, this result suggested that the length or the conformation of the activation loop was important for full activity, but apparently more so for polyE₄Y than for Src. This result suggested that although the activation loop was not part of the catalytic machinery, its conformation could have a dramatic effect on the activity of Csk in a substrate dependent manner.

Since none of the side chains in the activation loop was essential, we determined if we could remove all side chains from this region and retain Csk activity. An all-Gly-activation loop mutant (GAL), in which Gly replaced all 11 activation loop residues, was constructed. The mutant exhibited 1% and 14% of wt activity toward polyE₄Y and Src, respectively (Table 1). The differential phosphorylation of polyE₄Y and Src by GAL was
similar to Δ339, and further confirmed that the side chain functional groups were not required for catalysis, but the conformation of the loop affected the kinase activity in a substrate-dependent manner. Although the effects of the activation loop mutations were apparently substrate-dependent, only $k_{\text{cat}}$ values, but not the apparent $K_m$ values for the two substrates used were significantly affected. This suggested that phosphoryl transfer onto these substrates, instead of the recognition of these substrates by Csk was differentially affected by the activation loop mutations. The mutants displayed similar relative activity toward two other artificial substrates, RCM-lysozyme and GST fused to the optimal Csk substrate peptide, as toward polyE$_4$Y, suggesting that the mutants differentiated the physiological substrate from the artificial substrates.

**Csk is fully active without an intact activation loop.** Since the activation loop residues were not required for Csk function, we wanted to determine the effect of cleaving the activation loop. A mutant that contained a thrombin cleavage sequence in the activation loop (TC-Csk) was generated, in which the thrombin cleavage sequence of LVPRGS replaced ASSTQD in the activation loop. TC-Csk retained approximately 2% wt activity toward polyE$_4$Y and approximately 5% wt activity toward kdSrc (Table 1).

Fig. 4 shows the thrombin digestion on TC-Csk (Fig. 4A) and its effect on Csk activity (Fig. 4B). Incubation with buffer alone or thrombin did not cleave or affect the activity of wt Csk (data not shown). Incubation with buffer alone did not lead to cleavage of TC-Csk or significant change in its kinase activity compared to TC-Csk kept on ice. Incubation with thrombin led to cleavage of TC-Csk into two fragments at 38 and 12 kD, respectively, the expected sizes when the thrombin cleavage site was digested. This
cleavage activated TC-Csk, resulting in full recovery of the wt Csk activity toward both polyE4Y and kdSrc. This result indicated that the low activity of TC-Csk was due to a conformational effect, which could be released by thrombin cleavage, and that an intact activation loop was not required for full function of Csk.

The two fragments produced by thrombin digestion were both essential parts of the kinase core structure (Fig. 5A). It was surprising that the two parts could still make up a fully active enzyme. To confirm that the two fragments were indeed associated with each other after thrombin digestion, the digested enzyme was purified on a Ni\(^{2+}\)-IDA affinity column. Since only the 12 kD fragment contained the poly(His)_6 tag, co-purification of both fragments on a Ni\(^{2+}\)-affinity column would indicate that the two fragments were associated with each other. SDS-PAGE indicated that the relative ratios of the two fragments were similar before and after Ni\(^{2+}\)-affinity purification, thus confirming that the two fragments were strongly associated with each other (Fig. 5B). Examination of the crystal structure of Csk indicated that the two parts were held together by extensive hydrophobic interactions between the side chains on 3 \(\alpha\)-helices: \(\alpha\)E from the 38 kD fragment and \(\alpha\)F and \(\alpha\)I on the 12 kD fragments (Fig. 5A).

**Discussion**

PTKs of the Csk family are unusual in that they lack an autophosphorylation site in the activation loop, a regulatory mechanism conserved among all other PTKs. Considering the central location of this loop in the active site and well-demonstrated roles of autophosphorylation in the regulation of other PTKs, we probed the function of this loop in Csk with some surprising findings.
The activation loop of Csk is not essential for the kinase function. None of the mutations, including Ala scanning, loop replacement, site-specific deletion, replacing all residues by Gly, and cleaving the activation loop, completely abolished Csk activity. The lowest activity toward kdSrc was observed for TC-Csk, at 5%, but digestion of the activation loop with thrombin restored the activity completely. Similar recovery of kinase activity toward polyE₄Y was also observed, indicating that the low activity in TC-Csk, and likely in other mutants was not due to the loss of any functionalities in the activation loop, but an inhibitory conformation. These results indicated that the activation loop is not part of the catalytic machinery of Csk, nor is it required for the catalytic machinery to be fully functional.

Another important question is whether conformational changes in the activation loop do serve as a regulatory element in Csk, and if so, what regulatory signals can bring about such conformational changes. The SH3 and SH2 domains of Csk interact with and increase the activity of the catalytic domain (27-29). As discussed earlier, the active and full length Csk and inactive Csk lacking the regulatory SH2 and SH3 domains have major differences in the conformation of the activation loop (13, 14). It appears likely that interactions between the regulatory domains with the catalytic domain can affect the conformation of the activation loop. Whether conformational changes in the activation loop mediate the SH2-SH3 regulation of Csk awaits further investigation.

If the catalytic machinery between Csk and other PTKs are fundamentally the same, then these results suggested that the Csk activation loop was an evolutionally "basal" activation loop devoid of regulatory functionalities. The activation loops in other
PTKs acquired other functionalities that allow them to interact with and regulate the function of the catalytic machinery. Based on this perspective, we propose the following functionalities for the activation loop in other PTKs. First, the amino acid sequence itself may interact with the catalytic machinery and act as an inhibitor. Second, the amino acid sequence surrounding the Tyr residue is needed to render the Tyr residue a good substrate for the kinase. Third, the phosphorylated activation loop needs to form certain interactions so the inhibitory interactions are removed. Finally, the catalytic machinery and the activation loop may evolve in a corresponding fashion, so the potential regulation by the activation loop can be accommodated. Mutagenic and structural studies of insulin receptor kinase, which is regulated by autophosphorylation, are consistent with these perspectives (30-32). These perspectives may provide a baseline for dissecting autophosphorylation as a key mechanism for PTK regulation.

Consistent with this proposal, the catalytic machinery of Csk appears to be different from that of other PTKs. Arg313 is universally conserved among protein kinases (33) and the corresponding residue is essential in two PTKs that have been tested: mutations of the equivalent residue in Src (Arg385) (21) and v-Fps (Arg1042) (22) resulted in inactive (< 0.1% of wt activity) enzymes. But mutation of Arg313 had no effect on the Csk activity toward either the physiological or artificial substrates. This suggests the possibility that the role of this Arg residue has co-evolved with that of the activation loop: in PTKs that autophosphorylate, this Arg needs to interact with the phospho-Tyr for the kinase to be active, in PTKs that lack autophosphorylation, the catalytic machinery does not require the activation mediated by this residue. In Ser protein kinases, the equivalent residue also plays an important although not essential role.
Mutation of the corresponding Arg residue to Ala decreased the kinase activity by 90% in yeast cAMP-dependent protein kinase (Arg209) (34), and 80% in glycogen phosphorylase kinase (35, 36), indicating an important regulatory, but not a catalytically essential function for this residue.

*Some Csk activation loop mutants differentially phosphorylate the physiological and artificial substrates.* ∆339 and GAL exhibited only 1% and 2% of wt activity toward polyE₄Y but 14% and 18% wt activity toward kdSrc, indicating that the effect of the activation loop mutations are substrate-dependent. These observations raise an important question about the mechanisms Csk substrate recognition. Theoretically, there are two ways alterations in the activation loop could be propagated to other parts of the enzyme to affect the kinase activity. The first is by direct interaction of the side chains in this region. But Ala scanning and loop replacement results eliminated the possibility that the side chains in the activation loop are directly involved in regulation or catalysis. The second way the alteration can be propagated is by affecting the conformation of fragments that are covalently linked to the activation loop. The amino end of the activation loop is connected to the conserved DFG motif and the carboxyl end of the activation loop is linked to the P+1 loop. The DFG motif is catalytically essential as the mutation of Asp332Ala abolished the kinase activity toward both polyE₄Y and Src. The P+1 loop is responsible for interacting with peptide substrates (9). Propagation of the conformational changes to either DFG or P+1 loop would likely dramatically alter the kinase activity. Since the mutational effects were substrate-dependent, it suggested that the
conformational changes in the activation loop predominantly affected the function of the P+1 loop.

Then how do such conformational changes distinguish the artificial substrates from the physiological substrate? One explanation is that the phosphorylation of the artificial substrate predominantly relies on its interactions with the activation loop and P+1 loop while the phosphorylation of Src relies less on such interactions. This implies that Src also interacts with Csk in regions outside the activation loop/P+1 loop. This interpretation is consistent with our understanding of peptide substrate binding to PTKs (9) in general and consistent with the complex Csk-Src interactions that has been suggested by two lines of evidence. 1) Although Tyr527 in Src is an excellent substrate for Csk (18, 19), peptides mimicking the tail sequence of Src (19, 37, and our unpublished results) or heat inactivated Src (38) are much worse substrates compared to Src or to the optimal Csk peptide substrate, suggesting that Tyr527 recognition by Csk relies on more than just the local peptide sequence. 2) Mutations of the residues surrounding Tyr527 of Src have only relatively minor effect on the ability of Src to be phosphorylated by Csk (19). These observations indicate that some interactions that make Tyr527 of Src a good substrate for Csk are not located on the tail sequence surrounding the phosphorylation site, but in other regions of Src. Such a "bivalent Csk-Src" interaction model could provide a basis for the differential effects by the activation loop mutations. The nature and details of the Csk-Src interaction remains to be determined.
Acknowledgment:

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References


Figure Legends

Figure 1. Csk structure and the conformations of the activation loop. A. Crystal structure of full-length Csk showing the SH3 and SH2 domains, ATP-binding lobe (A-lobe), peptide-binding lobe (P-lobe), the catalytic and activation loops in the active site. B. Conformations of the activation loop in active (a) and inactive (b) Csk. The residues that align, such as Phe333, Thr336, are labeled together, and those that do not align, such as Asp332, Lys337, and Lys347, are labeled separately. Some residues in the activation loop were not resolved in the crystal structure and not shown. The alignment was performed by K2 protein structure alignment program (39).

Figure 2. Ala scanning mutagenesis of the Csk activation loop. A. Sequence of the Csk activation loop. The variable region to be studied is not underlined. B. Activity of Ala mutants using polyE4Y and Src as substrates. Asp332Ala and Arg313Ala are also included. Standard errors are shown. Invisible error bars indicate small errors to be seen in the graph.

Figure 3. Autophosphorylation and trans-phosphorylation of CSAL. A. Sequence comparison between Csk and Src activation loop. DFG motif, activation loop and the P+1 loop are indicated. The conserved residues are in bold. B. Csk and CSAL autophosphorylation and trans-phosphorylation by Src. Each lane contains 2 µg Csk or CSAL. 1 ng autophosphorylated Src is present in indicated lanes. C. Time course of Csk and CSAL phosphorylation by Src. Each lane contains 2 µg Csk or CSAL and 1 ng autophosphorylated Src.
**Figure 4.** Digestion of TC-Csk by thrombin. 20 µg Csk or TC-Csk were incubated with either buffer or 0.8 µg of thrombin (2 NIH units) for 2 h, and the digested proteins were analyzed by SDS-PAGE (A), or kinase activity assay using polyE<sub>4</sub>Y (closed bar) and kdSrc (open bar) as the substrates (B).

**Figure 5.** Hydrophobic interactions holding Csk fragments together. A. Thrombin digestion in the activation loop cleaves Csk into a 38 kD (in red) and 12 kD (in blue) fragments. Extensive hydrophobic interactions among the side chains from a helices E, F, and I likely hold the two fragments together. B. Co-purification of the two fragments by (His)<sub>6</sub> affinity chromatography. 1, TC-Csk; 2, Digested TC-Csk; and 3, digested TC-Csk after (His)<sub>6</sub> affinity chromatography.
Table 1. Kinetic parameters\(^a\) of wt and selected Csk mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(k_{cat} (E_{4Y}) ) (min(^{-1}))</th>
<th>(K_m (E_{4Y}) ) (µg ml(^{-1}))</th>
<th>(k_{cat} (kdSrc) ) (min(^{-1}))</th>
<th>(K_m (kdSrc) ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>34 ± 5</td>
<td>96 ± 22</td>
<td>113 ± 23</td>
<td>10 ± 0.5</td>
</tr>
<tr>
<td>E338A</td>
<td>6 ± 2</td>
<td>83 ± 17</td>
<td>49 ± 7</td>
<td>10 ± 1.6</td>
</tr>
<tr>
<td>Δ339</td>
<td>0.6 ± 0.04</td>
<td>106 ± 32</td>
<td>21 ± 10</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>GAL</td>
<td>0.3 ± 0.02</td>
<td>68 ± 9</td>
<td>16 ± 4</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>TC-Csk</td>
<td>0.7 ± 0.03</td>
<td>75 ± 13</td>
<td>6 ± 0.8</td>
<td>14 ± 0.4</td>
</tr>
</tbody>
</table>

Note:

\(^a\)Standard errors are presented. These values were calculated from three experiments, with each one performed in duplicates.
Figure 2.

A

DFGLTKEASSTQDTGKL

B

- polyE$_4$Y
- kdSrc

Relative activity (%)
Figure 3.

A

Csk: ---DFGLTK EASS TQD TGKL PVKWTAPEAL---
Src: ---DFGLARLIEDNEYTARQGAKFPIKWTAPEAA---

DFG motif Activation loop P+1 loop

B

Src: Csk:  wt CSAL wt CASL none
wt - - + + +

C

Csk or CSAL

Csk

CSAL

Time (min): 0 5 10 20 40 60
Figure 4.

Csk: wt wt TC TC TC
Treatment: none thrombin none buffer thrombin

MW (kD)

A

Full length
37 kD fragment
12 kD fragment

B

Relative kinase activity

wt TC TC TC
buffer thrombin
Figure 5.
Functions of the activation loop in Csk protein tyrosine kinase
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