Conserved hydrophobicity in the SH2–kinase linker is required for catalytic activity of Csk and CHK

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Abstract **The crystal structure of full-length Csk (C-terminal Src kinase) molecules shows a hydrophobic interaction between the SH2–kinase linker residue Phe183 and the αC-helix of the catalytic domain. To study the possible involvement of this contact in the regulation of the activity of Csk and CHK (Csk homologous kinase), a Csk SH2–kinase linker deletion mutant, Csk Phe183 and CHK Leu223 point mutants were analyzed. It was observed that a residue with a long hydrophobic side chain in position 183 (Csk) and 223 (CHK) is required to sustain the catalytic activity of Csk and CHK. These results suggest that Csk Phe183 and CHK Leu223 stabilize the movement of the αC-helix of these protein tyrosine kinases.**

Key words: C-terminal Src kinase; Csk homologous kinase; Site-directed mutagenesis

1. Introduction

Csk (C-terminal Src kinase) is a ubiquitously expressed 50 kDa protein tyrosine kinase, the main function of which is to downregulate the catalytic activity of Src-family kinases [1–3]. Together with CHK (Csk homologous kinase) [4] it belongs to the Csk-family of non-receptor tyrosine kinases. Csk is a modular protein consisting of SH3-, SH2- and kinase domains. However, Csk is unique among the tyrosine kinases because it lacks a conserved tyrosine autophosphorylation site in the kinase domain. Furthermore, Csk does not possess a myristylation site in the N-terminus or a regulatory phosphotyrosine site in the C-terminal tail.

Several studies have shown that the catalytic activity of Csk can be modulated to some extent [5–7]. The crystal structure of four active and two inactive full-length Csk molecules provides the molecular basis to study how control of Csk activity is achieved [8]. The crystal data show that in all Csk molecules, the SH3 and SH2 domains align on top of, but on opposite sides of the N-terminal lobe of the kinase domain. Furthermore, both the SH3–SH2– and the SH2–kinase linkers make extensive interactions with the kinase domain (Fig. 1A). The crystal data also show that in inactive Csk molecules, the SH2 domains are rotated upward, resulting in loss of some of the contacts between the SH2- and the kinase domain.

It is not precisely known which of the intramolecular interactions created by the SH2 domain, the SH3–SH2 linker and the SH2–kinase linker are essential for the catalytic activity of Csk or how these interactions regulate Csk activity. Thus far it has been shown that site-directed mutagenesis of SH3–SH2 linker residues decreases catalytic activity of Csk, indicating the importance of the SH3–SH2 linker for Csk activity [9].

The SH2–kinase linker of Src plays a critical role in the inactivation of Src. The side chain of Leu255, within the SH2–kinase linker, must be inserted into a hydrophobic pocket on the surface of the N-terminal lobe of the kinase domain to inactivate Src [10]. Interestingly, both Csk and CHK have in their SH2–kinase linker a residue with a long hydrophobic side chain at a position corresponding to Src Leu255 (Fig. 2A). These amino acid residues are Phe183 (Csk) and Leu223 (CHK), respectively. In the crystal structure of active and inactive Csk molecules, Phe183 lies within a short α-helix, designated αBC, and makes contact with the αC-helix in the N-terminal lobe of the kinase domain (Fig. 1A,B). Therefore, it is possible that conserved hydrophobicity in the SH2–kinase linker of Csk plays a role in the stabilization of the active conformation of the αC-helix.

In the present study, the role of conserved hydrophobicity in the SH2–kinase linker region as an aspect of the regulation of Csk-family kinases was analyzed. Deletion and site-directed mutagenesis revealed that the long hydrophobic side chain of Phe183 in Csk and Leu223 in CHK in the SH2–kinase linker region are required to maintain the full catalytic activity of Csk-family kinases. These data provide evidence that engagement of a hydrophobic SH2–kinase linker residue with the αC-helix is required for formation of a catalytically functional domain of the Csk-family kinases.

2. Materials and methods

2.1. Cell culture

HeLa tet-off cells (Clontech) expressing the tetracycline-controlled transactivator and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% (v/v), 2 mM L-glutamine and 100 U/ml penicillin – 100 μg/ml streptomycin (BioWhittaker).

2.2. Plasmid constructs

Csk constructs (Fig. 2B) were generated using either the Altered Sites in vitro Mutagenesis System (Promega) or the QuickChange site-directed mutagenesis method (Stratagene). In the Altered Sites Mutagenesis System mutations were introduced into human *csk* cDNA in the pALTER vector (Promega). Desirable mutations were confirmed by sequencing the relevant regions. The mutated Csk inserts were excised from pALTER vectors and subcloned into pUHD vectors [11] for expression in HeLa tet-off cells. In the QuickChange mutagenesis method mutations were introduced directly into human

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csk cDNA in the pUHD vector. CHK constructs (Fig. 2B) were generated using the QuickChange mutagenesis method and the pcDNA3FLAG-CHK vector [12] (a generous gift of Dr. Hava Avraham, Harvard Medical School, Boston, MA, USA)

2.3. Transfections, cell lysis and Western blotting

Wild-type (wt) and mutant pUHDCsk vectors were transfected into HeLa tet-off cells using FuGENE 6 according to the manufacturer's protocol (Roche Diagnostics) or the calcium phosphate method [13]. pcDNA3FLAG-CHK wt and mutant constructs were transfected into COS-1 cells using the calcium phosphate method.

After 48–72 h cell culture, transfected cells were scraped off the plates and disrupted in lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 mM $NaVO_4$, 50 mM NaF) for 20 min at 4°C. Undissolved material was removed by centrifugation of lysates for 15 min at 11 000×*g*. The protein concentration of cell extracts was determined using BCA Protein Assay (Pierce). Proteins were resolved in sodium dodecyl sulfate–polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked using 4% (w/v) bovine serum albumin (BSA) in Tris-buffered saline–Tween (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20). Specific proteins were detected using rabbit antiserum against Csk [14] or rabbit polyclonal antibodies against Csk (Santa Cruz Biotechnology), CHK (Lsk; Santa Cruz Biotechnology) and FLAG epitope (Sigma Aldrich) combined with horseradish peroxidase-linked secondary antibodies (Amersham Biosciences). Immunodetection was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech). To equalize the amounts of overexpressed kinases in immunoprecipitations, expression levels of Csk and CHK from immunoblots were quantified using Image Pro plus software (Media Cybernetics).

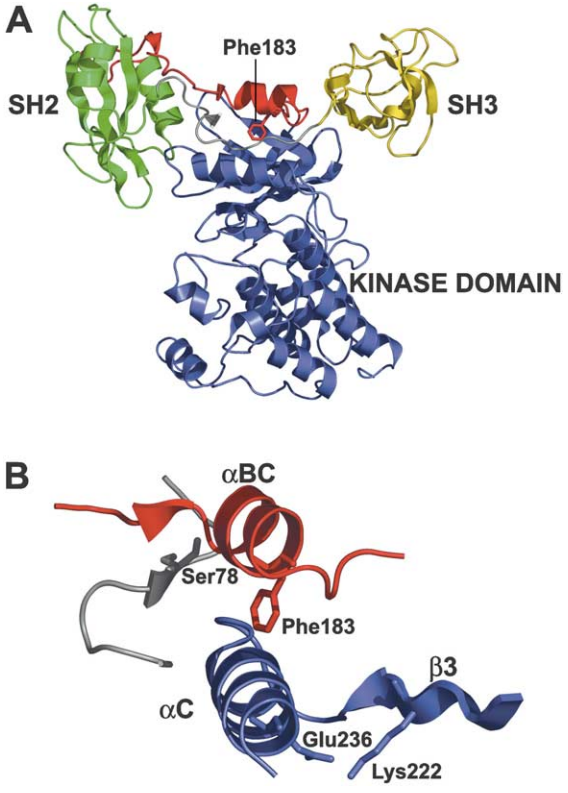

Fig. 1. A: Structure of active Csk (PDB entry 1K9A [8]) showing the location of Phe183. Domains and domain linkers are individually colored: SH3, yellow; SH3–SH2 linker, gray; SH2 domain, green; SH2–kinase linker, red; kinase domain, blue. B: The major contact of the SH2–kinase linker with the αC-helix is through Phe183. The SH3–SH2 linker (Lys72–Pro81), the SH2–kinase linker (Gly175–Leu190), the β3-strand and the αC-helix (Val219–Leu243) of the kinase domain are colored as in panel A. Side chains of Ser78, Phe183, Glu236 and Lys222 are shown in stick representation. Panels A and B were generated using PyMol [18].

2.4. Immunoprecipitations and kinase reaction

Equal amounts of overexpressed Csk and CHK were immunoprecipitated by specified antibodies. At least 500 μg of total protein was incubated with kinase-specific antibodies for 1 h at 4°C. Protein A–Sepharose (Amersham Biosciences) was added and incubation was continued for 0.5 h. Immunoprecipitates were centrifuged and washed three times with lysis buffer and twice with kinase buffer (10 mM HEPES pH 7.4, 5 mM $MnCl_2$). Kinase reaction was started by adding to the immunoprecipitates (25 μl bead volume) 5 μl kinase reaction buffer (10 mM HEPES pH 7.4, 5 mM $MnCl_2$, 5 mg/ml poly(Glu/Tyr) 4:1, 15 μM unlabeled ATP, 3 μCi [γ-^{32}P]ATP). Reactions were stopped after 15 min incubation at 30°C by adding 50 μl termination buffer (20 mM HEPES pH 7.4, 4 mM ATP, 40 mM EDTA, 2 mg/ml BSA). Four 10 μl aliquots of each sample were spotted onto filter paper squares (Whatman 3 MM) and labeled peptide substrate was precipitated on the filter paper by immersing the squares in 10% trichloroacetic acid – 8% sodium pyrophosphate. Unbound ^{32}P was removed by washing three times with 5% trichloroacetic acid and once with 95% ethanol. Radioactivity on the filters was quantified by liquid scintillation counting.

3. Results

3.1. Deletion of SH2–kinase linker residues Val177–Tyr184 decreases the catalytic activity of Csk

Shekhtman et al. have shown that the SH3–SH2 linker is essential for the catalytic activity of Csk [9]. To investigate whether the SH2–kinase linker is involved in regulation of the catalytic activity of Csk, residues Val177–Tyr184, within the SH2–kinase linker, were deleted. This deletion decreased the catalytic activity of mutant Csk to the same level as kinase-inactive K222R (Fig. 3A) and ΔSH3SH2. Because of the endogenous Csk in HeLa cells the reported weak catalytic activity of the Csk kinase domain (ΔSH3SH2) was not seen (Fig. 3A) [15].

3.2. Mutation of Phe183 to Ala decreases the catalytic activity of Csk

The crystal structure of Csk has revealed a direct association of the side chain of Phe183, located within the deleted segment Val177–Tyr184, with the αC-helix [8]. This suggests that Phe183 controls the orientation of the αC-helix and the catalytically critical Glu236 (Fig. 1B). To test this possibility, Phe183 was mutated to Ala. As shown in Fig. 3A, the F183A mutant was only slightly functional compared to Csk wt, suggesting that the association of the Phe183 hydrophobic side chain with the αC-helix is indeed crucial for the catalytic activity of Csk.

3.3. Mutation of Phe183 to Leu or Trp restores the catalytic activity of Csk

To test further the hydrophobic interaction between the side chain of Phe183 and the αC-helix, Phe183 was mutated to Leu and Trp. The results indicate (Fig. 3A) that the length and the size of the Phe183 side chain is an important factor in the interaction of the SH2–kinase linker with the αC-helix. Both mutations restored the catalytic activity of Csk, Leu serving a better substitution for Phe183 than Trp. Taken together these results demonstrate that the hydrophobic association of the SH2–kinase linker with the αC-helix via the indole ring of Phe183 is required to maintain the catalytic activity of the Csk kinase domain.

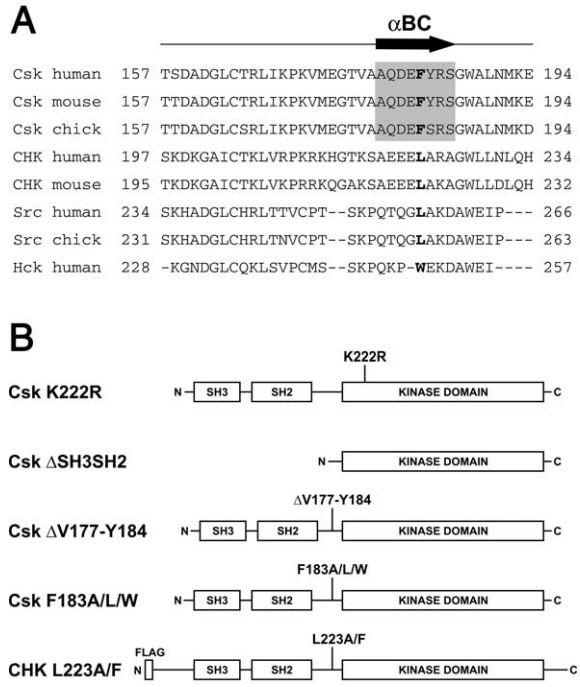

Fig. 2. A: Alignment of the SH2–kinase domain connecting sequences of Csk- and Src-family kinases. The αBC-helix in the SH2–kinase linker of Csk is indicated in light gray. Structurally equivalent residues to Src Leu255 are in bold. The sequences were aligned using CLUSTAL W [19] and the complete sequence of each kinase. B: Schematic representation of the deletion and point mutation constructs of Csk and FLAG-tagged CHK.

3.4. Effect of Leu223Ala and Leu223Phe point mutations on the catalytic activity of CHK

Because CHK has a leucine (Leu223) at a position corresponding to Csk Phe183, it was tested if the SH2–kinase linker is also involved in the regulation of CHK activity. As shown in Fig. 3B, mutation of Leu to Ala indeed decreased the catalytic activity of CHK. To demonstrate that the methyl side chain of Ala was too short to maintain the catalytic activity of CHK, Leu223 was mutated to Phe. As shown in Fig. 3B, this mutation restored the catalytic activity of CHK compared to the L223A mutant. Altogether these results suggest that the SH2–kinase linker is required to maintain the catalytic activity of the kinase domain of CHK.

4. Discussion

In the crystal structure of inactive Src, Leu255 within the SH2–kinase linker binds in a hydrophobic pocket on the surface of the N-terminal lobe of the kinase domain [16]. The interaction between Leu255 and the kinase domain leads to the rotation of the αC-helix and disruption of a salt bridge between Glu310 and Lys295. As a result, catalysis is inhibited because the displaced Glu310 can no longer align the side chain of Lys295 to coordinate the phosphate groups of ATP [10].

In contrast to Src, the SH2–kinase linker of Csk directly interacts with the αC-helix (Fig. 1B). This suggests that the SH2–kinase linker could control the movement of the αC-helix and therefore the formation of an ion pair between Glu236 (Src Glu310) and Lys222 (Src Lys295) (Fig. 1B). The decreased activity of Csk ΔV177-Y184 supports the idea that the contact of the SH2–kinase linker with the kinase domain is required to sustain the catalytic activity of Csk. However, the observed activity of the Csk ΔV177-Y184 mutant does not give conclusive evidence for the SH2–kinase linker involvement in Csk activity. Deletion of the SH2–kinase linker residues not only eliminates contacts made by the deleted residues but also results in the misplacement of the potential intramolecular interactions N-terminal to the deletion. Therefore the loss of these connections could also contribute to the observed activity of the Csk ΔV177-Y194 mutant.

Sequence alignment of Src and Csk SH2–kinase linkers reveals that Csk has a residue with a long hydrophobic side chain (Phe183) in a position corresponding to Src Leu255 (Fig. 2A). Phe183 lies within the αBC-helix and the crystal structure of Csk reveals a hydrophobic interaction of the Phe183 indole ring with the αC-helix (Fig. 1B). This contact suggests that the SH2–kinase linker interacts with the αC-helix via Phe183. Our studies confirm that the hydrophobic

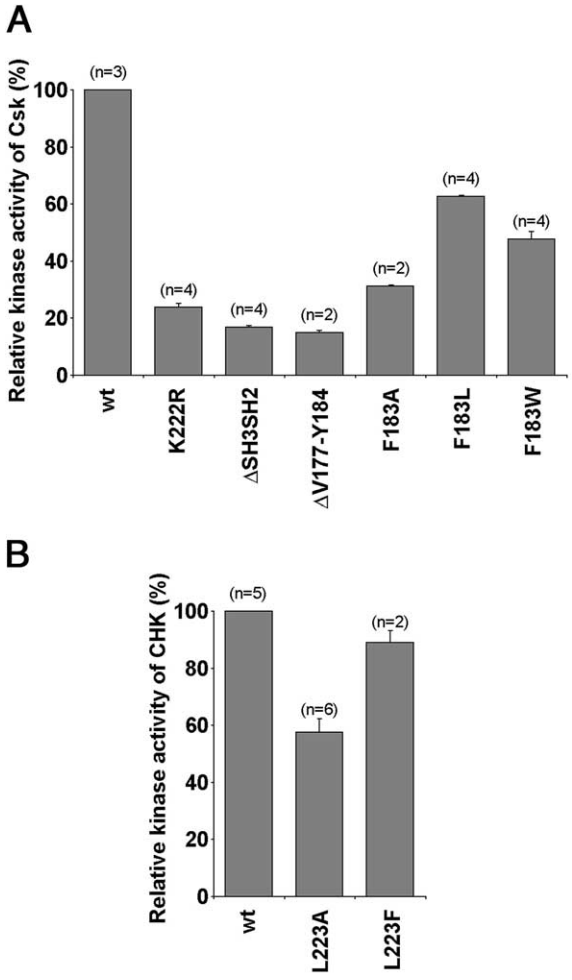

Fig. 3. A: Catalytic activity of overexpressed Csk SH2–kinase linker mutants in HeLa cells. The mutant Csk activity is shown as percentage of Csk wt activity with the number of determinations indicated in parentheses. Bars represent the standard deviation of the mean. B: Catalytic activity of overexpressed CHK SH2–kinase linker mutants in COS-1 cells. The mutant CHK activity is shown as percentage of CHK wt activity with the number of determinations indicated in parentheses. Bars represent the standard deviation of the mean.

interaction between Phe183 and the αC-helix is required to maintain the catalytic activity of Csk. Deletion of the Phe183 indole ring resulted in decreased activity, but the activity could be restored with a residue possessing a long hydrophobic side chain (Leu or Trp). The observed activity of the F183A mutant suggests that the loss of contact between the SH2–kinase linker and the αC-helix leads to a greater degree of freedom in the movement of the αC-helix and therefore to loss of functional orientation of Glu236. Consequently, the restoration of the activity of the F183L and F183W mutants indicates that the long hydrophobic side chains of leucine and tryptophan can make a contact with the αC-helix and control the orientation of the αC-helix. These results demonstrate that the orientation of the αC-helix regulates the catalytic activity of Csk and a critical parameter controlling the orientation of αC-helix is a hydrophobic contact between Phe183 and the αC-helix.

The crystal structure of full-length CHK is still unsolved. However, the close homology between CHK and Csk suggests that these kinases share a similar structure. Therefore, the engagement of the CHK SH2–kinase linker with the αC-helix could control the activity of the CHK kinase domain. Sequence alignment of the CHK and Csk SH2–kinase linkers supports this hypothesis (Fig. 2A). CHK has a Leu223 within the SH2–kinase linker in a position corresponding to Csk Phe183. This conserved hydrophobicity in the CHK SH2–kinase linker favors the possibility that CHK Leu223 is essential for catalytic activity of CHK, as Phe183 is for Csk. As suggested, deletion of the side chain of Leu223 decreased the catalytic activity of CHK, whereas activity could be restored by mutating Leu223 to Phe. Together these results indicate that Leu223 is necessary for the catalytic activity of the CHK kinase domain. The most probable explanation for the obtained results is that Leu223 makes a hydrophobic contact with the αC-helix and controls the orientation of the catalytically critical Glu276 (Csk Glu236), in a manner analogous to Csk Phe183.

Previous studies have indicated that mutation of the SH3–SH2 linker residues also decrease the catalytic activity of Csk [9,17]. The SH3–SH2 linker residue Ser78 was mutated in both studies. Because Ser78 makes contact with the SH2–kinase linker (Fig. 1B), it is possible, that the SH3–SH2 linker mutation results in weaker contact between linkers. Presumably a weak contact between the SH3–SH2– and SH2–kinase linkers allows a higher degree of movement of the αBC-helix on top of the N-terminal lobe of the kinase domain. As a result, catalytic activity decreases because Phe183 in the αBC-helix cannot contact the αC-helix to support the catalytically active conformation of the αC-helix.

In conclusion, our data indicate that, in contrast to Src, conserved hydrophobicity within the SH2–kinase linker is required to maintain the catalytic activity of Csk and CHK. These data provide evidence that interaction between the SH2–kinase linker and the αC-helix is required for formation of a functional catalytic domain of Csk-family kinases.

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