Activation of C-terminal Src kinase (Csk) by phosphorylation at serine-364 depends on the Csk–Src homology 3 domain

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In the present study, we investigate the mechanism for the protein kinase A (PKA)-mediated activation of C-terminal Src kinase (Csk). Although isolated Csk kinase domain was phosphorylated at Ser³⁶⁴ by PKA to the same stoichiometry as wild-type Csk, significant activation of the isolated Csk kinase domain by PKA was observed only in the presence of the purified Src homology 3 domain (SH3 domain). Furthermore, the interaction between the SH3 and kinase domains was facilitated by PKA-mediated

phosphorylation of the kinase domain, as evaluated by surface plasmon resonance. This suggests that an overall structural domain organization and interaction between the kinase and SH3 domains are important for the activity of Csk and its regulation by PKA.

Key words: C-terminal Src kinase, protein kinase A, Src homology 3, surface plasmon resonance, Src.

INTRODUCTION

Src family kinases (SFKs) are involved in a number of intracellular signalling processes regulating cell growth, proliferation and differentiation [1,2]. The activation state of SFKs is strongly dependent on the phosphorylation status of two tyrosine residues, one within the activation loop of the catalytic domain, and the other located at the C-terminus. Whereas the former acts as a positive regulator of kinase activity, the latter is phosphorylated by the C-terminal Src kinase (Csk) and mediates down-regulation of SFK activity [3–5]. Csk was originally purified and cloned from neonatal rat brain [4,5] and is expressed ubiquitously. Disruption of the *csk* gene in mice leads to neural tube defects and embryonic lethality, probably due to unregulated SFK activities in these embryos (increased by 5–15-fold) [6,7]. Furthermore, conditional inactivation of the *csk* gene in immature thymocytes gives rise to SFK hyperactivation (2–5-fold) and a concomitant pre-T cell receptor/ $\alpha\beta$ -T cell receptor-independent differentiation of $\alpha\beta$ T-lineage cells [8].

Although Csk is mainly cytosolic, a fraction of Csk partitions into lipid rafts due to interaction with a transmembrane adaptor molecule called Csk-binding protein (Cbp) or phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) [9,10]. Cbp/PAG is present exclusively in lipid rafts where a major pool of SFKs is located [9]. When phosphorylated at Tyr³¹⁷, Cbp/PAG binds the Src homology 2 domain (SH2 domain) of Csk, thereby recruiting Csk to lipid rafts and to the immediate vicinity of SFKs. Cbp/PAG is phosphorylated probably by SFKs [9–11], whereas results on the phosphatase(s) responsible for its dephosphorylation have not been published so far.

Domain organization in Csk is important for the overall regulation of Csk activity. The recent resolution of the crystal

structure of Csk [12] revealed both structural similarities and differences between Csk and SFKs. Although the kinase domain of Csk resembles that of an activated SFK [12,13], the positioning of the SH2 and SH3 domains relative to the kinase domain differs between SFKs and Csk [12,14,15]. The SH2 and SH3 domains in Csk reside on opposite sides of the N-lobe of the kinase domain and their peptide-binding pockets are both orientated outwards. The SH3–SH2 linker and the SH2–kinase linker form structures around which the SH2 domain pivots. An active conformation of the kinase domain seems to be dependent on the positioning of the SH2 domain relative to the kinase domain [12], which may explain the positive regulatory effect observed in Csk activity on ligation of the SH2 domain by tyrosine-phosphorylated Cbp/PAG [16]. Other reports have implicated the SH3 domain in overall regulation of Csk activity. Whereas deletion of the SH3 and SH2 domains in Csk lowers the specific kinase activity by approx. 100-fold, reconstitution studies with isolated domains suggest that the SH3 domain and not the SH2 domain is important for the conservation of Csk activity [17,18]. This effect of the SH3 domain is still present when the peptide-binding pocket is occupied, suggesting that other structures of the SH3 domain are important for stabilizing the kinase domain [17,18].

At least three different mechanisms are involved in the regulation of the phosphotransferase activity of full-length Csk. First, engagement of the Csk SH2 domain due to Cbp–PAG binding leads to a 2–4-fold induction of Csk activity [16]. Secondly, $G\beta\gamma$, probably due to binding to the catalytic domain of Csk, up-regulates Csk activity by 2-fold [19]. Thirdly, as we have demonstrated recently, Csk can be activated by covalent modification as well [20]. Protein kinase A (PKA) phosphorylates Csk on Ser³⁶⁴ both *in vitro* and *in vivo*, leading to a 2–4-fold

Abbreviations used: Csk, C-terminal Src kinase; Cbp, Csk-binding protein; GST, glutathione S-transferase; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; PKA, protein kinase A; RU, response unit; SFK, Src family kinase; SH domain, Src homology domain; SPR, surface plasmon resonance.

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Figure 1 Expression of various Csk mutants

Schematic representation (A) and Coomassie Brilliant Blue staining (B) of different Csk mutants expressed and purified from *E. coli*.

increase in Csk activity. In the present study, we have examined mechanisms for activation of Csk and show that PKA-mediated phosphorylation of this site increases the phosphotransferase activity of Csk by affecting an important interaction between the kinase and SH3 domains.

EXPERIMENTAL

Preparation of different Csk mutants

The construct encoding glutathione S-transferase (GST)–Csk [21] in the prokaryotic expression vector pGEX-KG was cut with restriction enzymes *Bpu*1102i and *Bam*H1 and subsequently re-ligated to yield a construct encoding GST–Csk domain (corresponding to amino acids 139–450 in Csk; Figure 1A). Csk kinase domain with His₆ tag on the N-terminus was obtained by cloning the Csk kinase domain into the *Nde*I–*Hind*III sites of pET28a (Novagen, Madison, WI, U.S.A.) using standard PCR technique and with pGEX-KG/GST–Csk as template. GST–Csk–SH3 and GST–Csk–SH3–SH2 were made by site-directed mutagenesis introducing a stop codon at amino acid positions 83 and 175 respectively (Figure 1A; Quickchange; Stratagene, La Jolla, CA, U.S.A.). The generation of Csk-Ser³⁶⁴-Cys was described elsewhere [20]. All constructs were confirmed by sequencing.

Expression and purification of proteins

Csk mutants were expressed in *Escherichia coli* BL21 as fusion proteins with GST, purified on glutathione–agarose beads, using

methods described previously [21–23] and isolated by cleavage from GST with thrombin [21–23]. Expression and purification of Csk kinase domain containing His₆ tag were performed as described elsewhere [24]. Milligram amounts of expressed and purified proteins (Figure 1B) were stored at –20 °C in 20 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA and 50% (v/v) glycerol for several months without loss of activity. Lck SH3–SH2 (catalogue no. sc-4037), Lck SH3 (catalogue no. sc-4038) and Grb2 SH3 (catalogue no. sc-4036) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Protein measurements

Proteins were quantified by the method of Bradford [25], using BSA as a standard, or by visual comparison of Coomassie Brilliant Blue-stained proteins and protein standards on SDS/PAGE [22].

Tyrosine kinase assays *in vitro*

The tyrosine kinase activity of Csk was measured as incorporation of phosphate into the synthetic polyamino acid poly(Glu, Tyr) 4:1 (Sigma) [21]. A standard procedure was followed with reaction volumes of 50 µl containing Hepes buffer (50 mM, pH 7.4), 5 mM MgCl₂, 100 µM ATP, 1.0–2.5 µCi of [γ -³²P]ATP, 200 µg/ml poly(Glu, Tyr) and 1 pmol of purified full-length Csk or 25 pmol of GST–Csk kinase domain or Csk kinase domain. When time courses were conducted, the reaction volumes were larger, typically 250 µl. Incubations were always at 30 °C for 12 min if not otherwise stated. At the end of the incubation, aliquots (35 µl) of each reaction mixture were withdrawn and spotted on to 10 mm × 30 mm strips of Whatman 3 MMTM chromatography paper, which were washed once in 10% (w/v) trichloroacetic acid/8% (w/v) sodium pyrophosphate for 15 min, twice in 5% trichloroacetic acid for 15 min and once in ethanol for 10 min, and finally counted with 4 ml of Opti-Fluor (Packard Bioscience B.V., Groningen, The Netherlands) scintillation fluid in a Liquid Scintillation Counter. Activity in the absence of poly(Glu, Tyr) was subtracted from all samples.

PKA-mediated phosphorylation of Csk

Csk mutants (2 pmol) were incubated at 30 °C for 20 min in the absence or presence of 5 pmol of PKA catalytic subunit C α , prepared as described previously [26], in a 20 µl volume containing 50 mM Hepes (pH 7.4), 200 µM ATP and 5 mM MgCl₂. When added, Csk–SH3 concentration was 3–30 µM. Reactions were stopped by the addition of SDS sample buffer, boiled and subjected to SDS/PAGE. Immunoblotting was performed (as described elsewhere [22]) using a polyclonal anti-phosphoserine-specific antibody towards PSer³⁶⁴ in Csk raised against a phosphopeptide and purified by multiple rounds of depletion of antibodies reactive to a dephospho peptide followed by affinity purification on a PSer³⁶⁴ peptide column.

Analysis of kinetic data

Estimation of K_d values for the interaction between the Csk kinase and SH3 domains was obtained by curve-fitting, using non-linear regression to fit the data to a rectangular hyperbola using the formula

$$y = f(x) = V_{\text{basal}} + V_{\text{increase}}[x/(K_d + x)]$$

where y is the activity of the Csk kinase domain at different values of x , x the concentration of Csk-SH3 domain, V_{basal} the basal activity of the Csk domain in the absence of Csk-SH3 domain and V_{increase} the incremental increase in the activity above basal levels when additional amounts of Csk-SH3 domains are added.

Surface plasmon resonance (SPR)

Studies to analyse the real-time interaction between Csk and Csk-SH3 domains were performed by SPR spectroscopy using Biacore 2000 and 3000 instruments (Biacore AB, Uppsala, Sweden). Here the interaction between an immobilized component, referred to as the ligand, and a molecule in the mobile phase, the analyte, is determined. Changes in surface concentration are proportional to changes in the refractive index on the surface resulting in changes in the SPR signal, plotted as resonance units (RU). 1000 RU corresponds to a surface concentration on the sensor chip of 1 ng of protein/mm² [27]. The method is described in detail elsewhere [28–30].

To investigate quantitatively the interaction of the kinase domain and the SH3 domain as analytes or ligands respectively, one partner had to be immobilized. Several strategies, including capture with specific antibodies either against the GST- or His₆-fusion part or against the Csk kinase domain, were tested for an active and stable immobilization. Only covalent coupling of the kinase domain was successful in immobilizing the Csk kinase domain using standard amine coupling [31] with an immobilization level of 4300 RU. Association and dissociation rates were determined by injecting a series of dilutions of the free SH3 domain or the GST-fusion SH3 domain for 2 and 5 min respectively at a flow rate of 30 μ l/min. All interaction studies were performed in running buffer [20 mM Mops/150 mM NaCl; pH 7.0] containing 0.005 % of surfactant P20 at 20 °C. After each interaction event, the kinase surface was regenerated by injecting 1 M NaCl in running buffer. Unspecific binding was subtracted using blank runs performed on an *N*-hydroxy-succinimide/*N*-ethyl-*N'*-(dimethylaminopropyl)-carbodi-imide-activated and ethanolamine-deactivated surface. Kinetic constants from the raw data were calculated by non-linear regression or equilibrium-binding analysis using the BIA evaluation software version 3.1 (Biacore AB). Several interaction models were studied. The non-linear regressions were fitted using global-fit analysis. Equilibrium-binding analysis was performed using the program Graphpad Prism using the equation

$$Y = B_{\text{max}} X / (K_d + X).$$

On-chip phosphorylation of the kinase domain by PKA

Amine-coupled Csk domain was phosphorylated with recombinant C subunit, prepared as described previously [26], for 1 h at 25 °C in running buffer (containing 1 mM ATP/5 mM MgCl₂) at a flow rate of 5 μ l/min.

Analytical gel filtration

Analytical gel filtration was performed using a Superose 12 HR 2/30 column on a Smart system (Amersham Pharmacia, Uppsala, Sweden) with a flow rate of 0.08 ml/min at 22 °C in running buffer A as described previously [32].

RESULTS

The Csk-SH3 domain is important for the phosphotransferase activity of Csk

To study mechanisms involved in PKA-mediated regulation of Csk, we first generated different Csk constructs (Figure 1A) that were expressed in *E. coli* and subsequently purified (Figure 1B). A comparison of the kinase activities of full-length Csk and the isolated Csk kinase domain (without SH3 and SH2 domains) revealed that the latter had an approx. 200-fold lower activity (223 ± 26 -fold lower, mean $\pm$ S.D., $n = 3$) (Figure 2 and results not shown). A GST fusion protein of the Csk kinase domain had even lower tyrosine kinase activity (625 ± 254 -fold lower, mean $\pm$ S.D., $n = 3$) (results not shown), which was in agreement with observations made by Sondhi and Cole [17]. Whereas the full-length enzyme showed a more or less linear time-dependent activity for 15–18 min (Figure 2, upper

Figure 2 Comparison of time-course kinase activities of wild-type Csk and Csk kinase domain

Accumulated phosphate transfer for indicated time periods were measured as incorporation of phosphate into poly(Glu, Tyr) as described in the Experimental section; activities were determined as mmol of phosphate transferred/mol of enzyme.

Table 1 Effect of Csk-SH3 domain on the catalytic activity of Csk-kinase domain in the absence and presence of catalytic subunit of PKA

The phosphotransferase activity of Csk kinase domain (0.5 μ M) was measured in the presence of various concentrations of Csk-SH3 (0–30 μ M) and in the absence and presence of C α (0.1 μ M) (see Figure 4 for one representative experiment).

[SH3] (μ M)	Activity (mmol $\cdot$ mol ⁻¹ $\cdot$ min ⁻¹)*		Fold induction by PKA†	Incremental increase (mmol $\cdot$ mol ⁻¹ $\cdot$ min ⁻¹)‡
	No PKA	With PKA		
0	9.6 $\pm$ 1.7	25.2 $\pm$ 3.1	2.6	15.6 $\pm$ 3.5
6	9.6 $\pm$ 0.9	54.9 $\pm$ 0.2	5.7	45.3 $\pm$ 0.9
10	11.2 $\pm$ 2.7	85.6 $\pm$ 7.1	7.7	76.0 $\pm$ 7.6
30	23.6 $\pm$ 5.0	184.4 $\pm$ 38.9	7.8	174.8 $\pm$ 39.2

* Absolute activities (means $\pm$ S.D.).

† Ratio of the activities in the presence and absence of PKA at a given SH3 concentration.

‡ Values represent increase in the activity, i.e. activity in the absence of Csk-SH3 is subtracted from activities obtained at different concentrations of Csk-SH3.

Figure 3 PKA-mediated phosphorylation of Ser³⁶⁴ in Csk is independent of the SH3 domain

(A) Demonstration of reactivity of antibody eluates from non-phospho and phospho-peptide columns towards different amounts of peptides. (B) Reactivity of P-Ser³⁶⁴-specific antibody towards different amounts of Csk phosphorylated by PKA, as described in the Experimental section. (C) Csk-wt or Csk mutants (0.1 μ M) were incubated with or without catalytic subunit (0.25 μ M C α) of PKA in the presence of Mg-ATP as described in the Experimental section. When added, the concentration of Csk-SH3 was 8 μ M. After SDS/PAGE, immunoblotting with a rabbit polyclonal antiserum specific towards P-Ser³⁶⁴ in Csk was performed.

part), a decline in the activity of the Csk kinase domain was observed after <5 min of incubation (Figure 2, lower part). Given the low phosphotransferase activity of the Csk domain, longer incubation periods were necessary to obtain reproducible and consistent measurements, typically 10–12 min. Since deletion of the SH3 and SH2 domains of Csk impaired its specific activity, we next examined the effect of adding purified Csk-SH3-SH2 or SH3 domains on the activity of the isolated kinase domain. Csk-SH3-SH2 or SH3 domains were both individually capable of restoring the activity of the Csk domain, but only partially. At concentrations up to 10 μ M, neither Csk-SH3 nor SH3-SH2 domains were capable of increasing the activity of the isolated kinase domain. However, at higher concentrations (up to 30 μ M) both Csk-SH3 and SH3-SH2 domains had stimulatory effects on the activity of the kinase domain, with the SH3 domain being approximately twice as efficient (data on SH3 in Table 1, Figure 4; results on SH3-SH2 not shown). This suggests an important role for the SH3 domain in the regulation of the kinase domain of Csk. Further studies

Figure 4 Importance of SH3 domain in the optimal activation of Csk kinase domain by PKA

Phosphotransferase activity of Csk kinase domain (0.5 μ M) was measured in the presence or absence of C α (0.1 μ M) and different concentrations of Csk-SH3 as described in the Experimental section. Phosphotransferase activities were measured as incorporation of phosphate into poly(Glu, Tyr) as described in the Experimental section; activities were determined as mmol of phosphate transferred $\cdot$ (mol of enzyme)⁻¹ $\cdot$ min⁻¹. The upper curve is obtained by curve-fitting using non-linear regression as described in the Experimental section, and the lower curve is a linear fit.

Figure 5 Interaction of Csk-SH3 with the kinase domain immobilized to a sensor chip

(A) Csk kinase domain (4300 RU) was immobilized covalently to CM 5 sensor chip using amine coupling. Association and dissociation rates were determined by injecting a series of dilutions of the free SH3 domain from 34 μ M to 67 nM for 2 min at a flow rate of 30 μ l/min (curves represent 34, 17, 8.6, 4.3, 2.1, 1.1, 0.54, 0.27, 0.13 and 0.067 μ M SH3 from top to bottom respectively). (B) Steady-state analysis of the interaction in (A). A nonlinear regression analysis (one-side binding hyperbola) yielded a K_d value of 9.1 ± 0.75 μ M.

Figure 6 Comparison of nonlinear regression analysis for a series of dilutions of Csk-SH3 on phosphorylated Csk kinase domain

(A) Interaction of Csk-SH3 with unphosphorylated (---) and phosphorylated Csk kinase domain (—). SH3 domain was injected as described in Figure 5(A) over the surface using the concentrations indicated in the Figure. After on-chip phosphorylation with PKA catalytic subunit (mouse $C\alpha$), the same concentrations of SH3 domain were injected, clearly displaying a different binding pattern. (B–F) Global-fit analysis of the experiment in Figure 5(A); only one concentration ($34 \mu\text{M}$) is shown. (B) Langmuir 1 : 1 model was applied, yielding good fits for the interaction of Csk-SH3 on unphosphorylated Csk kinase domain (results not shown; Table 2), but not for the biphasic-binding behaviour of the Csk-SH3 on phosphorylated Csk interaction. (C) Two-state conformational change model of SH3-kinase interaction. (D) Interaction studied applying a model assuming bivalent analyte (i.e. Csk-SH3). (E) The model heterogeneous ligand parallel reaction shows much better fits when compared with that in (B–D), indicating that two species of ligand, the phosphorylated and the unphosphorylated Csk, were present simultaneously on the chip surface. (F) A dissection of the kinetics fitted for the model in (E), displaying a set of fast and slow rate constants respectively (see Table 2).

revealed that the activity of the Csk kinase domain was not affected by purified SH3 or SH3-SH2 domains from Lck or Grb2 SH3 domain (results not shown). Additionally, Abl SH3 domain has been shown previously to have no impact on the isolated Csk domain. Altogether, this indicates that the observed effects were specific for the Csk SH3 domain as observed elsewhere [17,18].

PKA phosphorylates Csk on Ser³⁶⁴ independent of the Csk-SH3 domain

We have described previously that Csk is phosphorylated at Ser³⁶⁴ by PKA, and that this phosphorylation increases the tyro-

sine kinase activity of Csk by 2–4-fold. Therefore we investigated whether PKA was capable of phosphorylating and thereby activating the isolated Csk kinase domain, and we explored the effects of the SH3 domain on this activation. A polyclonal phospho-specific antibody towards P-Ser³⁶⁴ in Csk was raised and characterized (Figures 3A and 3B). As expected, full-length Csk-wt was phosphorylated by PKA, whereas mutant Csk-Ser³⁶⁴-Cys was not (Figure 3C, left panel). The isolated Csk kinase domain was also phosphorylated effectively, and addition of purified SH3 domain did not affect the stoichiometry of the PKA-mediated phosphorylation of Ser³⁶⁴ of the Csk kinase domain (Figure 3C, right panel). These results indicate that the Csk protein was folded properly and recognized as a substrate for PKA.

Table 2 Rate and equilibrium-binding constants for Csk-SH3 domain interaction with phosphorylated and unphosphorylated Csk kinase domains

Model	Csk-SH3 on unphosphorylated kinase			Csk-SH3 on phosphorylated kinase		
	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_d (μM)	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_d (μM)
Equilibrium analysis	n.a.*	n.a.	9	n.a.	n.a.	12
Langmuir 1:1	3.2×10^4	2.9×10^{-1}	8.9	Bad fit	Bad fit	n.a.
Heterogeneous ligand parallel reaction	n.a.	n.a.	n.a.	$K_{d1} = 3.2 \times 10^4$ $K_{d2} = 5.8 \times 10^4$	$K_{d1} = 2.7 \times 10^{-1}$ $K_{d2} = 3.1 \times 10^{-3}$	$K_{d1} = 8.3\ddagger$ $K_{d2} = 5.3\ddagger$

* n.a., not accessible.

† Fast phase (unphosphorylated-kinase domain).

‡ Slow phase (phosphorylated-kinase domain).

The impact of PKA-mediated phosphorylation on Csk phosphotransferase activity is potentiated by the Csk-SH3 domain

The catalytic activity of Csk kinase domain incubated with PKA was only 2-fold higher than that of the kinase domain alone, and the absolute value of the corresponding increase in activity was almost negligible, due to very low basal activity (Figure 4, open circles; Table 1). Interestingly, PKA-mediated activation of Csk kinase domain was substantially stronger in the presence of purified Csk-SH3 protein, both with respect to relative increase and in absolute numbers (Figure 4, Table 1). In the presence of PKA, the induction in activity increased in a concentration-dependent manner with increasing concentrations of SH3 domain, and at higher concentrations the effect appeared saturable (Figure 4, filled circles). An estimated K_d for the interaction between the kinase and SH3 domains was $34.0 \pm 11.4 \mu\text{M}$ (mean $\pm$ S.D., $n = 3$). In comparison, the activity of the kinase domain increased in a curvilinear, almost linear, manner with increasing concentrations of SH3 domain in the absence of PKA (Figure 4, open circles), and the effect did not seem to be saturable even at very high concentrations of the SH3 domain. This may be due to a lowering of the affinity between Csk and SH3 domains in the absence of PKA-mediated phosphorylation of Ser³⁶⁴, and curve extrapolation suggested that the K_d value for this interaction was $> 150 \mu\text{M}$ (based on several experiments). In addition, PKA phosphorylation also potentiates the stimulatory effect of the SH3 domain on the kinase domain. Whereas increasing amounts of SH3 domain up-regulate the activity of the kinase domain up to 2.5-fold in the absence of PKA, the corresponding number in the presence of PKA is > 8 -fold (Table 1). Thus phosphorylation of Ser³⁶⁴ probably affects both K_d and V_{max} values (Table 1).

PKA-mediated phosphorylation of the Csk domain affects its interaction with the SH3 domain

We next examined the SH3-kinase domain interaction by SPR. Figure 5(A) shows interaction of Csk-SH3 domain with unphosphorylated Csk kinase domain immobilized to a sensor chip. Association and dissociation rates were determined by injecting a series of concentrations of the free SH3 domain (67 nM–34 μM) or the GST-fusion SH3 domain (results not shown) for 2 min at a flow rate of 30 $\mu\text{l}/\text{min}$. This demonstrated that there was an interaction between the two domains, and a steady-state analysis of the interaction yielded a K_d value of 9 μM (Figure 5B). To study changes in the binding pattern by PKA phosphorylation of Csk kinase domain, we phosphorylated the kinase domain on the sensor chip (as described in the Experimental section) and injected 4.3–34 μM SH3 domain. Significantly, higher binding levels were achieved when the kinase domain was phosphorylated (Figure 6A). Four different models

were tested for the analysis of the interaction of phosphorylated-kinase domain with the SH3 domain using global-fit analysis (Figures 6B–6E). Langmuir 1:1 model (Figure 6B) yielded good fit for the interaction between Csk-SH3 and unphosphorylated-kinase domains, giving a K_d value of 8.9 μM (Table 2); however, this model did not result in a good fit for the biphasic-binding pattern of Csk-SH3 to phosphorylated Csk kinase domain. Next the two-state conformational change and bivalent analyte (i.e. Csk-SH3) models were used, but did not fit very well (Figures 6C and 6D). Finally, using a model assuming heterogeneous ligand in parallel reaction, an excellent curve fit was performed (Figure 6E). Calculation of the association and dissociation rate constants yielded equilibrium-binding constants of 8.3 and 5.3 μM for the fast and slow phases respectively (Table 2). The fit parameters of the model 'heterogeneous ligand parallel reaction' indicate that two species of ligand, the phosphorylated and unphosphorylated kinase domains, were present simultaneously on the chip surface, i.e. that the Csk kinase domain was not phosphorylated to a stoichiometry of 1.0. Dissection of the kinetics fitted for the model in Figure 6(E) displayed a set of fast and slow rate constants for the two-species ligand (Figure 6F, Table 2). The presence of unphosphorylated kinase domain was also demonstrated by comparing the fast-phase dissociation constant in the heterogeneous ligand parallel reaction with the Langmuir 1:1 model (Table 2). Table 2 summarizes the association (k_{ass}) and dissociation (k_{diss}) rate constants and the equilibrium-binding constants (K_d) for the interaction of unphosphorylated or phosphorylated Csk kinase domain with SH3 domain.

DISCUSSION

In the present study, we have examined the molecular mechanism of the PKA-mediated activation of Csk as described recently [20]. Kinetic studies with recombinant proteins revealed that deletion of the SH3 and SH2 domains of Csk decreased its kinase activity by approx. 200-fold, and as shown by Sondhi and Cole [17], addition of SH3 and not SH2 domain was needed to increase the kinase activity. By adding increasing amounts of purified SH3 domain, we demonstrated a concentration-dependent increase in the activity of the isolated kinase domain. The addition of Csk-SH3 also augmented the activating effect of PKA on the isolated Csk kinase domain, despite not affecting the stoichiometry of PKA-mediated phosphorylation of Csk kinase domain at Ser³⁶⁴. Furthermore, the affinity for the interaction between Csk kinase and SH3 domains increased on PKA phosphorylation, and even more during the interaction time. This suggests that kinase-SH3 domain interaction is required for an efficient activation of Csk by phosphorylation at Ser³⁶⁴. This interaction is probably

intramolecular in the intact Csk, since the addition of soluble SH3 domain was not sufficient to compensate completely for the loss of activity by truncation of Csk.

At the present time, it is not clear why the isolated Csk kinase domain displays such a low catalytic activity. Our results suggest that a domain–domain contact between the Csk kinase and SH3 domains promotes the activity of the Csk kinase domain, perhaps through an allosteric mechanism. The observation that PKA phosphorylated both full-length Csk and the Csk kinase domain to very similar stoichiometry indicates that the SH3 domain is not necessary for the PKA-mediated phosphorylation of the kinase domain. Since the SH3 domain strongly augmented the activation of the Csk kinase domain, even when present as a separate protein, it appears that probably both phosphorylation and the SH3 domain stabilize the same catalytically active conformation of the kinase domain. This notion is supported by the change in $V_{\max}$ value for the interaction between Csk kinase and SH3 domains.

The observation that the SH3 domains from other proteins when compared with Csk did not increase the activity of Csk kinase domain, indicates specificity of the SH3 domain of Csk. Two recent studies have examined in detail the interaction between the Csk kinase domain and the SH3 domain and probed the nature of the SH3-binding surface involved. It was shown that neither a proline-rich peptide from the PEST (Pro, Glu, Ser, Thr)-enriched protein tyrosine phosphatase, which binds the SH3 domain of Csk, nor mutagenesis of the SH3-binding pocket affected the interaction of the SH3 domain with the kinase domain [17,18]. We conclude that the SH3 domain interacts with the kinase domain without using its type II helix ligand-binding surface. This is also consistent with the recently described crystal structure of Csk, indicating that the binding pocket of the SH3 domain is orientated outwards [12]. In agreement with a different mode of SH3–kinase interaction, engagement of the SH3-binding pocket (e.g. by PEST-enriched protein tyrosine phosphatase) has not been shown to affect the activity. In contrast, the Csk–SH2 domain, by itself, does not seem to regulate the activity in the same way, but when a ligand, such as tyrosine-phosphorylated Cbp/PAG, occupies the SH2 domain, this may increase the kinase activity by facilitating an active conformation of the kinase domain [16].

Assessment of the equilibrium-binding constants by SPR for the interaction of the Csk–SH3 domain with the phosphorylated and unphosphorylated kinase domains respectively was distinct but in the same range ($K_{d1} = 5.3$ and $K_{d2} = 8.9 \mu\text{M}$). However, calculation of the apparent rate constants demonstrated a major difference in the interaction of the phosphorylated and unphosphorylated Csk. The association rate constant of the fast phase in both the phosphorylated and the unphosphorylated interactions are identical, assuming a model where two different species of ligands, i.e. the phosphorylated and the unphosphorylated kinase domains, are immobilized and a homogeneous analyte (i.e. SH3) population is injected, yielding two parallel reactions (model heterogeneous ligand parallel reaction; see Table 2 and Figure 6A). These values fit very well with an equilibrium analysis using steady-state conditions and calculating binding constants for rate constants based on a 1:1 Langmuir model (see Table 2). An additional phase is detected when the kinase is phosphorylated on the chip, yielding higher response levels with the same concentrations of SH3 injected. Although the equilibrium-binding constant comparing phosphorylated and unphosphorylated kinase domains is not affected significantly either under steady-state conditions (12 versus $9 \mu\text{M}$, Table 2) or from evaluation of the association and dissociation rates directly (8 and $5 \mu\text{M}$), the apparent rate constants for the parallel reactions differ

by two orders of magnitude (Table 2, Figure 6F), indicating that the time in associated and dissociated states may be longer when Csk is phosphorylated. Furthermore, taking into account that the SH3 and kinase domains are in the same chain, i.e. with zero degrees of freedom, these effects of changes in the association time may be even more significant in the wild-type Csk.

In summary, we have examined the mechanism for PKA-mediated activation of Csk via phosphorylation of Ser³⁶⁴. We have demonstrated a mechanism by which phosphorylation of Ser³⁶⁴ affects the intra-chain interaction of the SH3 and kinase domains in Csk and thus regulates the activity by increasing $V_{\max}$ values of the enzyme.

Note added in proof (received 1 April 2003)

While this paper was in review, paper was published showing that CD45 dephosphorylates Cbp/PAG upon T cell activation [33].

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