Identification of Csk tyrosine phosphorylation sites and a tyrosine residue important for kinase domain structure

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The lack of a conserved tyrosine autophosphorylation site is a unique feature of the C-terminal Src-kinase, Csk, although this protein tyrosine kinase can be autophosphorylated on tyrosine residues *in vitro* and in bacteria. Here we show that human Csk is tyrosine phosphorylated in HeLa cells treated with sodium pervanadate. Phosphorylation *in vivo* occurs mainly at Tyr-184 and *in vitro* mainly at Tyr-304. A Y304F mutation strongly decreased Csk phosphorylation *in vitro*, and a Y184F mutation abolished tyrosine phosphorylation *in vivo*. A catalytically inactive form of Csk was also phosphorylated on Tyr-184 *in vivo*, suggesting that this is not a site of autophosphorylation. The kinase activity of the Y184F protein was not changed, while the Y304F protein showed one-third of wild-type activity. Three-

dimensional modelling of the Csk kinase domain indicated that the Y304F mutation abolishes one of two conserved hydrogen bonds between the upper and the lower lobes in the open conformation of the kinase domain. Phosphopeptide binding studies suggested that phosphorylation of Tyr-184 creates a binding site for low-molecular-mass proteins. Cellular Csk was associated with several phosphoproteins, some of which were interacting with the Csk SH2 domain. Taken together these results indicate that Csk can be phosphorylated *in vivo* at Tyr-184 by an as yet unknown tyrosine kinase, and that autophosphorylation of Tyr-304 occurs only at abnormally high Csk concentrations *in vitro*. Furthermore, Tyr-304 is required for the maintenance of the structure of the Csk kinase domain.

INTRODUCTION

The Csk kinase (C-terminal Src-kinase; [1,2]) belongs to a small family of cytoplasmic protein tyrosine kinases (PTKs) that includes the murine Ctk/Ntk [3,4] and human HYL/Matk/Lsk kinases [5–7]. The Ctk/Ntk and HYL/Matk/Lsk kinases show very high sequence identity and probably represent products of alternative splicing of transcripts of human and mouse homologues of the same gene, whereas Csk is clearly a distinct gene product. These kinases have been shown to have the capacity to phosphorylate the conserved C-terminal tyrosine residue of several tyrosine kinases of the Src family (Tyr-527^{c-Src} [3,4,8,9]).

The selective phosphorylation of the C-terminal regulatory site of Src-family kinases keeps them inactive by creating a phosphotyrosine residue that interacts with their SH2 (and SH3) domains [10]. The ability of Csk to down-regulate Src kinases has also been shown *in vivo* in *csk*^{−/−} mice, which had elevated Fyn activity in tissues [11,12]. Recently we have shown that a fraction of Csk is localized in focal adhesions of cultured cells via its SH2 domain, and that increased tyrosine phosphorylation occurs in the focal adhesions of HeLa cells overexpressing wild-type (wt) Csk. Interestingly, overexpression of active Csk in these cells led to a drastic rearrangement of a vitronectin receptor ($\alpha_v\beta_3$) and gross rearrangements of cell morphology. These changes appeared to occur without detectable changes in endogenous Src activity, suggesting that Csk has other substrates [13].

A hallmark of the Src family of PTKs is auto(trans)-phosphorylation of a highly conserved tyrosine residue, located in the

catalytic domain. This phosphorylation is required for full activity of these PTKs [14]. Several other PTKs, including growth factor receptors, require such transphosphorylation for activation by ligand-induced dimerization [15]. However, the tyrosine residue corresponding to the canonic autophosphorylation site of tyrosine kinases (Tyr-416^{c-Src}) is missing in Csk [2,16], and in cells expressing normal levels of Csk, phosphorylated forms of this enzyme have not been detected. Thus the possible regulation of Csk functions by phosphorylation has not been reported.

SH2 domains bind phosphorylated tyrosine residues in proteins. For example, (auto)phosphorylation sites of several receptor tyrosine kinases have been shown to function as docking sites for proteins involved in signal transduction. The current view is that SH2 domains serve as binding sites between proteins that function in adjacent steps of signal transduction pathways (reviewed in [17]). To this end, we and others have shown that the SH2 domain of Csk can interact with tyrosine-phosphorylated forms of the focal adhesion kinase and paxillin [13,18], and Rafnar et al. [19] have shown interaction of the Csk SH2 domain with the T-cell receptor ζ and ϵ chains. However, in most cells expressing normal levels of Csk, proteins associated with the kinase are not readily detected. In particular, Sabe et al. [20] have noted that cellular c-Src is not associated with Csk.

Protein kinases, both serine/threonine- and tyrosine-specific, contain several conserved or invariant residues [21], which have been shown to be structurally and functionally crucial. The structural scaffolding is similar in all known protein kinases, as indicated by the crystal structures of the protein serine/threonine

Abbreviations used: Csk, C-terminal Src-kinase; PTK, protein tyrosine kinase; cAPK, cAMP-dependent protein kinase; IRK, insulin receptor PTK; tet, tetracycline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; mAb, monoclonal antibody; HRP, horseradish peroxidase; PAS, protein A–Sephacrose; PGS, protein G–Sephacrose; TLE, thin layer electrophoresis; PDB, Protein Data Bank; GST, glutathione S-transferase; wt, wild-type; Fmoc, fluorenyl-9-methoxycarbonyl.

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kinases, cAMP-dependent protein kinase (cAPK; [22,23]), Cdk2 [24], twitchin kinase [25], MAP (mitogen-activated protein) kinase ERK2 [26], casein kinase-1 [27] and the insulin receptor PTK (IRK) [28]. Despite very limited sequence identity, all these kinases have the same bi-lobal fold. The upper domain is formed by an antiparallel β -sheet and one or two α -helices, and the lower lobe predominantly by α -helices. The upper region is rotated relative to the lower lobe in the active, closed, conformation. The phosphate-donating ATP molecule is bound by conserved residues in a cleft between the two lobes. Mutations in several locations are known to inactivate kinases and cause diseases, the structural consequences of which have been elucidated based on three-dimensional information, e.g. in [28–31].

Here we show a tyrosine-phosphorylated form of over-expressed Csk, which binds several phosphoproteins in HeLa cells. The main tyrosine phosphorylation site *in vivo* was identified, and the phosphorylation *in vivo* was shown not to be autocatalytic. Studies of the main phosphorylation site *in vitro* led to the discovery of a tyrosine residue crucial for the maintenance of a functional catalytic domain of Csk. Our data further suggest that some of the phosphoproteins found associated with Csk might be its substrates.

EXPERIMENTAL

Cell culture and radioactive labelling

The experiments were performed using HeLa cells expressing wt or different mutant variants of human Csk under the control of a tetracycline (tet)-responsive promoter that is silent in the presence and active in the absence of tet [13,32]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum (FCS) in the absence or presence of 1 μ g/ml tet. HtTA cells [32], expressing the tet-binding *trans* activator protein tTA, were transfected with the different Csk expression constructs using the calcium phosphate precipitation method [33]. The different Csk proteins were expressed either transiently or in stable cell lines. For metabolic 32 P-labelling, cells were incubated in DMEM containing 5% (v/v) FCS dialysed against 150 mM NaCl and 1–2 mCi of [32 P]P_i/ml for 6 h. For 35 S-labelling, cells were incubated in methionine/cysteine-free, serum-free DMEM containing a 100 μ Ci/ml methionine/cysteine mix (Promix; Amersham) for 6–8 h.

Plasmid constructs

All the Csk proteins studied in HeLa cells were expressed from pUHD10-3 vectors [13,32]. These constructs were designated pUHDCskwt, pUHDCskK222R [13], pUHDCskY184F, pUHDCskY304F and pUHDCskY403F. The point mutations were introduced into the *csk* cDNA using the pALTER system (Promega), except for the K222R mutation which was created by a PCR strategy [13]. The Tyr $\rightarrow$ Phe mutant cDNAs synthesized from pALTER were introduced into the pUHDCskwt construct by exchange of appropriate restriction fragments. The glutathione S-transferase (GST-CskSH2) and SH3 domain fusion proteins were produced as described previously [13].

The wt Csk used in kinase assays *in vitro* was produced from the baculovirus transfer vector pVL1392Csk in Sf9 insect cells and purified as described previously [10].

Immunoprecipitations and Western blotting

For immunoprecipitation and Western blotting, the rabbit anti-Csk C-terminal antiserum [9] was used and tyrosine-phosphorylated proteins were detected with the anti-phosphotyrosine monoclonal antibody (mAb) PY20 (Affiniti Research). For

Western blotting, cells were lysed in TKB lysis buffer [20 mM Tris/HCl, pH 7.5/150 mM NaCl/5 mM EDTA/1% (v/v) Nonidet P40/1 mM sodium orthovanadate (Na_3VO_4)/1 mM PMSF/aprotinin (20 mg/ml)]. The protein concentration of the cleared lysates was determined with the BCA Protein Assay Reagent (Pierce). Per lane 10–15 μ g of protein was analysed in SDS/7.5% or 10% polyacrylamide gels and blotted on to nitrocellulose filters using a semi-dry blotting apparatus (Ancos). The transfer efficiency and protein amounts were checked by Ponceau staining of the filters, which were then blocked in 4% BSA, probed with the different primary antibodies and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The specific proteins were detected using chemiluminescence.

For immunoprecipitation, cells were lysed in TKB lysis buffer. For co-immunoprecipitation and phosphorylation studies, cells were incubated in 0.1 mM sodium pervanadate (0.1 mM Na_3VO_4 /2 mM H_2O_2) at 37 °C for 20 min before lysis. Lysates were adjusted to equal protein concentrations (approx. 3 mg/ml), pre-cleared with Protein A-Sepharose (PAS; Pharmacia) for anti-Csk immunoprecipitations or Protein G-Sepharose (PGS) for anti-pY immunoprecipitations and then antibodies were added. After 2–3 h incubation on ice, PAS or PGS was added and the slurry was rolled end-over for 30 min. The Sepharose resins were then washed three times with TKB and twice with 10 mM Tris/HCl (pH 7.4). In some cases the resins were additionally washed twice with TKB containing 0.5 M NaCl before the final TKB and Tris/HCl washes and PAGE. To remove phosphate from serine and threonine, some gels were treated with 1 M KOH at 55 °C for 1 h.

Enzyme assays

The activity of p50^{csk} was measured in immunoprecipitates from TKB-lysates using a polyE/Y assay as previously described [13].

Tryptic and CNBr cleavage and phosphopeptide mapping

For tryptic phosphopeptide analysis, pure *in vitro* 32 P-labelled Baculo Csk or *in vitro* or *in vivo* 32 P-labelled immunoprecipitated Csk was run in SDS/PAGE and the wet gel was subjected to autoradiography. A 50 kDa band was cut out from the gel and the polypeptide was electroeluted using a BIOTRAP device (Schleicher & Schuell) according to the manufacturer's protocol. The protein was precipitated using trichloroacetic acid, dried, digested with trypsin and analysed by two-dimensional phosphopeptide mapping using pH 1.9 buffer for electrophoresis (88% formic acid/acetic acid/water, 25:78:897, by vol.) and isobutyric acid buffer for chromatography (isobutyric acid/n-butanol/pyridine/acetic acid/water (625:19:48:29:279, by vol.) [34]. CNBr cleavage of trypsin-digested Csk was carried out using 75 mg/ml CNBr (Eastman Kodak) [34,35].

Phosphoamino acid analysis

To determine if radioactively labelled Csk, *in vitro* or *in vivo*, was phosphorylated on tyrosine, serine or threonine, either intact immunoprecipitated Csk or tryptic fragments of Csk were hydrolysed and analysed by thin layer electrophoresis (TLE). In the case of intact Csk, electroeluted and trichloroacetic acid-precipitated protein was used, while for tryptic fragments, labelled spots were scraped from TLC plates and their contents eluted in the pH 1.9 buffer. The plate material was removed by centrifugation, the buffer evaporated and both types of samples were resuspended in 6 M HCl and subjected to phosphoamino acid analysis as described in [34].

Manual Edman degradation

Trypsin- or trypsin- and CNBr-cleaved phosphopeptides were scraped from TLC plates and eluted in the pH 1.9 buffer. The phosphopeptides were vacuum dried, redissolved in 30 μ l of 50 % (v/v) acetonitrile, and spotted on an arylamine-Sequelon disc (Millipore). The immobilized peptides were subjected to manual Edman degradation [36] with subsequent detection of radioactivity released at each cycle by liquid scintillation counting.

Peptide assays

Twelve amino acid Csk peptides containing the Tyr-184 residue were synthesized in a phosphorylated and an unphosphorylated form, and in a version containing a phenylalanine instead of tyrosine at position 184 (*N*-DEFY/pY/F/RSGWALNM-C). As an irrelevant peptide control, a random phosphopeptide (*N*-pYSWEGNFDMLAR-C) was used. A 433A Peptide Synthesizer (Applied Biosystems) and fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry was used. The resin-bound peptides were synthesized on TentaGel SNH₂ resin with a base label linker (Rapp Polymers) in a mix of TentaGel S RAM (10 %) to obtain peptide amides on an acid label linker for analyses of the synthesis products. Phosphorylated forms of tyrosine were obtained by addition of Fmoc-Tyr(PO₃Me₂)-OH (Bachem). The accuracy of synthesis and the composition of the synthesis products were confirmed in mass spectroscopy using Lasermatt. The peptides were kindly provided by Dr. Hilkka Lankinen (Haartman Institute Peptides, Helsinki, Finland). The concentration of the bound peptides was approx. 0.4 ng of peptide/ μ g of resin.

For the affinity assays, ³⁵S-methionine/cysteine-labelled cells were lysed in buffer [50 mM Hepes (pH 7.5)/150 mM NaCl/10 % (v/v) glycerol/1 % (v/v) Triton X-100/1 mM EGTA/1.5 mM MgCl₂/100 mM NaF/10 mM sodium pyrophosphate/1 mM Na₃VO₄/1 mM PMSF/aprotinin (20 mg/ml)]. Lysates were pre-cleared with uncoupled resins, after which the samples were incubated at 4 °C with 40 μ l of a 10 % (w/v) slur of resin-coupled peptides for 2 h, with continuous mixing. The pellets were then washed six times with the above lysis buffer and three times with 150 mM NaCl/50 mM Tris/HCl, pH 7.4. In peptide competition experiments, uncoupled peptides were added to the lysates before addition of the resin-coupled peptides, and the lysates were mixed for 2 h.

Molecular modelling

The Csk kinase domain was modelled based on the structure of the IRK at 2.1 Å resolution [28] {Protein Data Bank (PDB) [37], entry 1irk}. The sequence alignment was performed with GCG [38] and MULTICOMP program packages [39]. The model was built with the program InsightII (Biosym Technologies, San Diego, CA, U.S.A.). A side-chain rotamer library was used to model substitutions. Deletions and insertions were modelled by searching a database of high-resolution molecules from either most of the PDB structures or an unbiased selection of the PDB [40,41]. The model was refined by energy minimization with the program CHARMM [42] (version 23) using the all-hydrogen parameter set 22 (Chemistry Department, Harvard University, Cambridge, MA, U.S.A.) in a stepwise manner using an adapted basis Newton-Raphson algorithm. First, hydrogen atoms were relaxed, then the borders of indels were harmonically restrained and the rest of the molecule was fixed. In the next step the borders of indels and the C α atoms of the conserved regions, and finally only the C α atoms of the conserved regions, were harmonically constrained. The hydrogen bonds were analysed by using program InsightII, which employed distance and stereo-

chemical criteria, and the final model was tested using several criteria [44,45]. The co-ordinates for the open conformation of cAPK were taken from PDB entry 1atp [43].

RESULTS

Csk autophosphorylation *in vitro* and tyrosine and serine phosphorylation *in vivo*

In order to analyse Csk autophosphorylation *in vitro*, purified Baculo Csk was incubated for different periods of time with ATP and subjected to Western blotting using anti-pY and anti-Csk antibodies. This analysis showed tyrosine phosphorylation of Csk, associated with a retardation of the mobility of the Csk polypeptide in SDS/PAGE. The reaction levelled off at about 2 h of incubation (Figure 1). We next set out to determine if Csk, like most known tyrosine kinases, was phosphorylated also in mammalian cells. However, Csk could not be detected by anti-pY blotting of whole cell lysates, anti-Csk immunoprecipitates or anti-pY immunoprecipitates (results not shown) using the previously described inducible Csk expression system, which yields 15–20-fold elevated levels in HeLa cells [13].

For a more sensitive analysis of Csk phosphorylation, the incorporation of ³²P into immunoprecipitated Csk in kinase reactions *in vitro* or in orthophosphate-labelled HeLa cells was studied. In order to inhibit protein tyrosine phosphatases, the cells were treated with sodium pervanadate for 20 min before lysis. One of the major phosphoproteins detected in wt Csk immunoprecipitates in both cases had a molecular mass of 50 kDa and co-migrated in gels with pure Csk phosphorylated *in vitro* (Figure 2A). Interestingly, a 50 kDa phosphoprotein was also seen in immunoprecipitates from cells expressing a kinase-deficient form of Csk, K222R (Figure 2A, lane K222R) but not in kinase assays *in vitro* and no labelled co-precipitated proteins could be detected using this mutant (Figure 2B, lane K222R). However, phosphoproteins of 33, 36 and 76 kDa were associated *in vivo* with both wt and K222R Csk, while phosphoproteins of 23/24 and 31 kDa were associated with only wt Csk. The latter proteins as well as the pp76 were also phosphorylated in kinase reactions *in vitro* using wt Csk immunoprecipitates (Figure 2A). To check if pp60^{c-Src} was among the associated proteins,

Figure 1 Csk is phosphorylated on tyrosine *in vitro*

Pure Csk was incubated in the absence and presence of ATP, and samples of the reactions were analysed by anti-pY Western blotting (upper panel). Anti-Csk staining of the same blot (lower panel) shows that the more slowly migrating protein represents a highly phosphorylated form of Csk (arrows).

Figure 2 Detection of a 50 kDa phosphoprotein and Csk-associated phosphoproteins in Csk-overexpressing HeLa cells

(A) Autoradiogram of Csk immunoprecipitates from metabolically labelled control H1TA cells (C) and cells overexpressing the inactive CskK222R or wt Csk, compared with *in vitro* labelled immunoprecipitated (wt k.r.) and Baculo Csk. Pure pp60^{c-Src} was included in one sample (Src). All samples were run in the same gel. The immunoprecipitates were washed with lysis buffer containing 0.5 M NaCl and the gels were treated with KOH. Note that several phosphoproteins are associated with both wt and K222R Csk and become phosphorylated in the kinase reaction *in vitro*. (B) Csk is associated with several phosphoproteins through its SH2 domain. Csk immunocomplexes from pervanadate-treated control H1TA cells (C), cells overexpressing kinase inactive CskK222R or wt Csk were used for kinase reactions *in vitro*. Immunoprecipitations from equal amounts of cell lysates were performed in the absence (—) or in the presence of 1 nM of GST–CskSH2 or SH3 domain fusion proteins, or GST alone. (C) The 50 kDa phosphoprotein is Csk. Immunoprecipitation of electroeluted, labelled, 50 kDa phosphoprotein (A, lane wt k.r.), using pre-immune serum, immunogen-blocked anti-Csk antiserum or anti-Csk antiserum. Only the anti-Csk antiserum precipitates the 50 kDa protein.

Baculo pp60^{c-Src} was added to a kinase reaction with Baculo wt Csk. As can be seen in Figure 2(A) in the immunoprecipitates (compare lane Src), no phosphoprotein of the expected size was detected.

To analyse the regions of Csk involved in binding the co-precipitated phosphoproteins, purified GST–CskSH2 and GST–CskSH3 fusion proteins were added to lysates before immunoprecipitation and kinase reactions. These competition experiments showed that the association of a protein of 31 kDa and a protein doublet of 23–24 kDa was completely competed by addition of free CskSH2 domain and to some degree by the SH3 domain, whereas GST had no effect (Figure 2B).

The phosphorylation of pure Csk (Figures 1 and 2A) suggested that the kinase was autophosphorylated *in vitro*. To prove that the pp50 detected in *in vitro* kinase reactions with Csk immunoprecipitates was indeed phosphorylated Csk and not a co-migrating protein, this phosphoprotein was electroeluted from the gel and immunoprecipitated with Csk antiserum. As seen in Figure 2(C), pp50 was precipitated by the Csk antiserum, but not by pre-immune serum or by antiserum blocked using the immunogen peptide.

Phosphoamino acid analyses were carried out after partial

Figure 3 Phosphoamino acid analysis of Csk

In vitro phosphorylated, pure Baculo Csk and Csk immunoprecipitated from H1TA cells are shown in the upper panels. The lower panels show analysis of wt Csk and CskK222R immunoprecipitated from [³²P]P_i-labelled cells. *In vitro* labelled Csk is phosphorylated only on tyrosine, whereas *in vivo* phosphorylation occurs on both tyrosine and serine. (Pi denotes free phosphate).

hydrolysis of ³²P-labelled pure or immunoprecipitated Csk labelled in a kinase reaction *in vitro*, or Csk metabolically labelled with [³²P]P_i. The results showed that autophosphorylation of Csk *in vitro* occurred exclusively on tyrosine, while labelled Csk *in vivo*, both wt and the K222R form, contained both phosphoserine and phosphotyrosine (Figure 3).

Taken together these experiments show that Csk is capable of autophosphorylation on tyrosine residues *in vitro*, and that Csk phosphorylation occurs on both tyrosine and serine residues *in vivo*. The phosphorylation of the K222R mutant *in vivo* implicated another kinase. Furthermore, three phosphoproteins (23, 24 and 31 kDa) associated with the Csk SH2 domain and were phosphorylated, presumably by Csk, in *in vitro* kinase reactions.

Identification of the tyrosine residues phosphorylated in Csk

Similar tryptic two-dimensional phosphopeptide maps, showing two major spots (marked 1 and 2), were obtained from pure Csk and Csk phosphorylated in the immunocomplex reaction (Figures 4A and 4B). However, *in vivo* labelled Csk showed only one major spot (Figure 4C), which co-migrated with spot 1 when pure *in vitro* and *in vivo* labelled Csk were mixed (Figure 4D). The phosphopeptide map of *in vivo* labelled kinase-deficient Csk also contained spot 1 (Figures 4E and 4F). These experiments showed that Csk contains two major sites of phosphorylation, one phosphorylated only *in vitro* and the other both *in vivo* and *in vitro*.

Because the level of phosphorylation of Csk *in vivo* was low and because the tryptic peptide of spot 1 migrated identically in the analyses both *in vitro* and *in vivo*, *in vitro* labelled pure Csk was used for determination of the phosphorylated tyrosine residue. Spot 1 was eluted and subjected to Edman degradation. This analysis showed release of radioactive phosphate from the tryptic peptide at cycle 13 (Figure 5A). The only tyrosine in Csk located 13 amino acids from an arginine or a lysine residue is Tyr-184, which therefore is the main *in vivo* site.

Because we were unable to detect release of radioactivity after Edman degradation of the peptide of spot 2, the analysis was performed after additional cleavage by CNBr. The two-dimensional peptide map of this sample contained four spots (results not shown). Edman degradation of the two major

Figure 4 Two-dimensional tryptic phosphopeptide mapping of phosphorylated Csk

(A) *In vitro* phosphorylated pure Baculo wt Csk. The main spots are denoted 1 and 2 (Pi denotes free phosphate, and the circle at the bottom of the panel shows the origin). (B) Immunoprecipitated wt Csk phosphorylated in a kinase reaction *in vitro*. (C) and (E) wt and K222R Csk respectively immunoprecipitated from ^{32}P -metabolically labelled cells. (D) Mixture of the samples in (C) and (A). (F) Mixture of the samples in (E) and (A). Note that spot 2 is missing in the *in vivo* labelled Csk [compare (A) and (C)]. Also note that the peptide in spot 1 is phosphorylated in both wt Csk and CskK222R and co-migrates with spot 1 in (A).

spots released radioactivity at cycles 11 and 2 respectively. Release at cycle 11 was compatible only with Tyr-184 (results not shown). However, the release of radioactivity at cycle 2 matched either Tyr-304 or Tyr-403 (Figure 5B).

Analysis of phosphorylation of Y → F Csk mutants

In order to prove that the identified sites of phosphorylation indeed were used *in vitro* and *in vivo*, and to distinguish between Tyr-304 and Tyr-403, both were mutated to phenylalanine along with Tyr-184. The mutant Csk cDNAs were then expressed in HtTA cells and tryptic phosphopeptide maps were compared. This analysis showed that the mutation Y184F abolished spot 1 of both *in vivo* (Figures 6A and 6B) and *in vitro* labelled Csk (Figures 6D and 6E). The Y304F mutation abolished phosphorylation of spot 2 of *in vitro* labelled Csk (Figure 6F), but, as expected, did not change the pattern of phosphopeptides of *in vivo* labelled Csk (Figure 6C). Instead, the signal from another phosphopeptide was increased (Figure 6F, upper spot) possibly due to a compensatory *in vitro* phosphorylation. Finally, the Y403F mutation did not affect phosphorylation (results not shown). The phosphorylation of Tyr-184 and Tyr-304 was confirmed by analysis of CNBr cleavage products of the *in vitro* labelled mutant proteins. This analysis, in which the entire samples were analysed using SDS/PAGE, not including any steps where peptides could be lost due to different solubility or

Figure 5 Csk is mainly phosphorylated on tyrosine 184 *in vivo*

(A) Edman degradation of the peptide eluted from spot 1 (Figure 4) of trypsin-digested, *in vitro* phosphorylated Baculo Csk. The diagram shows radioactivity released at each degradation cycle. Below the numbers of degradation cycles is shown the stretch of the amino acid sequence of Csk that fits with the tryptic site (arrow). (B) Edman degradation of a phosphopeptide eluted from the major spot produced by trypsin and CNBr double cleavage of labelled Baculo Csk. The two possible tyrosine residues in Csk fitting the degradation/release data are shown. The sites of cleavage by trypsin and CNBr are indicated by arrows.

other properties, clearly showed that Tyr-304 was the main *in vitro* site (results not shown). Thus Tyr-184 is the preferred site of Csk phosphorylation *in vivo*, while both Tyr-184 and Tyr-304 are autophosphorylated *in vitro*. The positions of these tyrosine residues are shown schematically in the lower part of Figure 6.

Kinase activity of the Y → F Csk mutants

The kinase activity of the Y → F mutants was compared with that of wt and kinase-deficient Csk using *in vitro* kinase assays. While the activity of the Y184F mutant was indistinguishable from the wt protein, the Y304F mutant consistently had lower activity (about one-third of wt activity) (Table 1). Since autophosphorylation of wt Csk before the kinase assay did not affect Csk activity (results not shown), and since Tyr-304 was not a phosphorylation site *in vivo*, the reduction of the activity of Y304F Csk was apparently caused by some mechanism other than phosphorylation.

Analysis of the three-dimensional model of the Csk kinase domain

The most plausible reason for the impaired activity of the Y304F mutant protein was structural. To analyse this possibility, the structure of the kinase domain of Csk was modelled based on the IRK structure [28].

Analysis of the model revealed that residue Tyr-304 was located in the α -helix E on the surface and that it presumably

Figure 6 Two-dimensional tryptic phosphopeptide maps of Y → F mutant Csk proteins

(A) *In vivo* phosphorylated, immunoprecipitated wt Csk (note spot 1). (B) Y184F mutant protein. Note the total absence of signal. (C) Y304F map showing phosphorylation of spot 1, corresponding to Tyr-184. (D–F) Immunoprecipitated wt, Y184F and Y304F Csk labelled in kinase reactions *in vitro*. The lower part of the Figure shows a schematic presentation of the locations of the phosphorylated tyrosines in Csk.

Table 1 Relative activity of different forms of Csk

Shown are the results of poly-E/Y phosphorylation assays using immunoprecipitated wt, Y304F, Y184F and K222R Csk from five different experiments. Equal expression of the different forms was confirmed by Western blotting. The activity is shown relative to the endogenous Csk activity of HiTA cells, set as 1. Note the reduction of the activity of the Y304F mutant kinase, compared with wt.

HiTA	1
wt	14.55 ± 1.93
Y304F	4.67 ± 0.89
Y184F	14.00 ± 1.78
K222R	0.86 ± 0.07

formed a hydrogen bond with Arg-244 from helix C (Figure 7). There are only a few interactions between the upper and the lower lobe. In addition to bonds to ATP and the essential Mg²⁺ ions, there are only six hydrogen bonds in the closed conformation of cAPK, two of which are present also in the open conformation (results not shown). The long C-terminal end of cAPK, wrapping around the lower lobe, was excluded from this comparison, because the corresponding part is missing from other kinases. Those same two hydrogen bonds are conserved also in the open conformation of IRK and in the model of the Csk structure. Both these hydrogen bonds (Tyr-304–Arg-244 and Asn-247–Val-330) are shown in Figure 7. Thus a probable

reason for the low activity of the Csk Y304F protein is that the phenylalanine cannot form the hydrogen bond required to fix the upper and lower lobes.

Phosphorylated Tyr-184 binds cellular proteins

Since phosphorylation of Tyr-184 did not appear to be involved in the regulation of Csk catalytic activity, we studied if this tyrosine could be part of a recognition motif for, e.g., SH2 domain-containing proteins. TentaGel resin-bound peptides of 12 amino acids were used, containing either an unphosphorylated or a phosphorylated Tyr-184 residue, or a phenylalanine in this position. A control phosphopeptide containing the same 12 amino acids as the Tyr-184 phosphopeptide in a random order was also included. Neither the unphosphorylated peptide, the phenylalanine peptide, nor the random phosphopeptide bound specific proteins (Figure 8, lanes 2, 8 and 10). However, the phosphopeptide bound proteins of approx. 33, 27, 25 and 23 kDa, the 33 kDa protein being the most abundant (Figure 8, lanes 3, 7 and 9). These associations were specifically competed off when free phosphopeptide was added to the methionine/cysteine-labelled HeLa cell lysates before precipitation, but not when unphosphorylated peptide was added (Figure 8, lanes 4, 5 and 6). Anti-pY immunoblotting of the precipitates showed that the peptide-associated proteins were not phosphorylated on tyrosine (results not shown). Thus the function of phosphorylation of Tyr-184 may be to create a binding site for other proteins.

Figure 7 A three-dimensional model of the Csk kinase domain

The ribbon runs along the polypeptide chain backbone. α -Helices are in red and β -strands in dark blue. The positions of the conserved hydrogen-bond-interacting Arg-244 and Tyr-304 are indicated and shown in yellow. Another hydrogen-bonded amino acid pair in conserved positions, Asn-247 and Val-330, are shown in the centre of the structure in magenta.

DISCUSSION

Most PTKs studied so far show autocatalytic activity, and phosphorylation on tyrosine has been shown to be an important regulatory mechanism either of the kinase activity or for association of the kinases with other proteins [15,17]. Here we investigate if such mechanisms regulate the functions of Csk, which lacks the Src-type autophosphorylation sites in its kinase domain. Our results show that a phosphorylated form of both wt and kinase-deficient K222R Csk can be immunoprecipitated from HeLa cells treated with the protein tyrosine phosphatase inhibitor sodium pervanadate. Phosphorylation of Csk occurs at low stoichiometry on tyrosine and serine *in vivo*. Since Csk is strictly tyrosine-specific, the serine phosphorylation must be catalysed by another kinase.

In contrast with the *in vivo* situation, Csk autophosphorylation is readily detected in kinase reactions *in vitro* using pure Csk or wt Csk immunoprecipitates from HeLa cells. Our tryptic mapping and CNBr-cleavage analyses of the different mutant proteins showed that both Tyr-184 and Tyr-304 can be phosphorylated, but that Tyr-304 was the preferred site *in vitro*. Thus we conclude that Csk can be autophosphorylated *in vitro* at high concentrations, in agreement with previous observations [46–48]. The aberrant Tyr-304 autophosphorylation *in vitro* might be explained by the accessible location of this residue on the outer surface of the E helix of the kinase domain (Figure 7). Tyr-184 could also be accessible to autophosphorylation *in vitro*, since this residue is located in the probably flexible ‘hinge region’ between

Figure 8 Association of low-molecular-mass proteins with a phosphotyrosine-184-containing peptide

Affinity precipitations using different forms of a 12 amino acid peptide from methionine/cysteine-labelled HtTA cell lysates. Lane 1, a Csk immunoprecipitate analysed for comparison. Lane 2, affinity precipitation using the unphosphorylated peptide (P^-). Lane 3, precipitation with the phosphorylated Tyr-184 peptide (P^+). Note that one major protein of 33 kDa and proteins of approx. 27, 25 and 23 kDa are specifically associated with the peptide (arrows). Lanes 4 and 5 show competition experiments in which 30 nM (lane 4) or 300 nM (lane 5) of free phosphorylated peptide was added to the lysates before precipitation with the resin-bound P^+ peptide. Note the strong reduction of binding of the proteins. Lane 6 shows that the P^- peptide (300 nM) does not compete for the binding of the Csk associated proteins. Lanes 7 and 8, a separate affinity precipitation experiment with the phenylalanine-containing peptide (P^F), as compared with the P^+ peptide. Lanes 9 and 10 show that a random phosphopeptide (P^R) does not interact with the low-molecular-mass proteins seen using the P^+ -184 peptide.

the SH2 and TK domains. However, *in vivo*, only Tyr-184 was found to be phosphorylated. This event occurs *in trans*, since also the kinase-deficient form of Csk was Tyr-184 phosphorylated to approximately the same extent as the wt enzyme.

The difference between our results *in vivo* and *in vitro* could be due to an irrelevant reaction *in vitro* or an inhibition of this reaction *in vivo*. For example, the autophosphorylation of c-Abl PTK has been suggested to be prevented by an inhibitor *in vivo* [49]. Alternatively, Csk could be very efficiently dephosphorylated *in vivo*. Since Csk is autophosphorylated in *Escherichia coli* [46], such inhibition might not function in bacteria. However, this hypothetical inhibitor appears to operate not only in mammalian cells, but also in yeast and in insect cells, where phosphorylated Csk could not be detected by anti-pY blotting, despite high levels of expression [10], and results not shown).

The main site of Csk phosphorylation *in vivo* was shown to be Tyr-184, located in the short sequence linking the kinase and SH2 domains. This location suggested that phosphorylation of Tyr-184 was unlikely to regulate the Csk catalytic activity, a prediction confirmed by our mutagenesis data. Importantly, Tyr-184 was also phosphorylated *in vivo* in the kinase-deficient form of Csk, strongly implicating another kinase. Experiments *in vitro* using pure Csk and Src (Figure 2) and co-expression of these two kinases from baculoviruses in insect cells (wt Src plus wt or K222R Csk) indicated that the kinase phosphorylating Tyr-184 is not Src (M. Bergman, unpublished work).

The sequence surrounding Tyr-184 does not fit any known sequence interacting with SH2 domains [50,51]. Nevertheless, the phosphopeptide affinity precipitation experiments suggested that phosphorylation of Tyr-184 might create a binding site for low-molecular-mass proteins, in particular for one of 33 kDa. These proteins were not recognized by antibodies against pY, c-Crk, Shc or Grb2 (results not shown). Also, they were not phosphorylated in kinase reactions of affinity precipitates *in vitro* and they were apparently too small to accommodate a kinase domain. In contrast, they could contain SH2 or PTB domains and could thus be adapter-type molecules [17].

Recently, binding of a 36 kDa protein to Csk was described in HEL and K562 cells. This p36 was shown to be phosphorylated on tyrosine and to bind to the SH2 domain of Csk [52]. In our co-immunoprecipitations from *in vivo* labelled cells a phosphoprotein of about 36 kDa was also detected (Figure 2A), but no protein of that size was detected in kinase reactions of Csk immunoprecipitates *in vitro* (Figure 2B). Instead, 23, 24 and 31 kDa phosphoproteins were bound to Csk and competed by its SH2 domain (Figure 2B). These proteins as well as the previously described pp36 may be substrates of Csk, because they were phosphorylated both *in vivo* and *in vitro* in wt Csk but not in K222R Csk expressing cells (Figures 2A and 2B). Supporting this assumption, these proteins became phosphorylated when pure Csk was added to Csk immunoprecipitates from K222R Csk overexpressing cells (results not shown). The properties of these proteins are under further investigation.

Studies of effects of overexpression of Y184F Csk in HeLa cells showed some qualitative differences compared with expression of the wt enzyme. Y184F cells became less rounded than wt cells, less Csk was found in focals, and the vitronectin receptor rearrangement, seen in wt Csk-overexpressing cells [13], was less pronounced (results not shown). These findings support the assumption that some protein-protein interactions might be absent in the cells expressing Y184F Csk.

The model of the Csk kinase domain was constructed based on IRK. It should be noted that modelling of several protein kinases [29,53] has been carried out based on the remarkable structural homology of the catalytic core of the protein kinase family. Eight crystal structures of protein kinases have confirmed the overall structural conservation of the core. All major changes, insertions and deletions, were located in loops on the protein surface. There were five deletions and one insertion. The model was finished with energy minimization and evaluated with several techniques.

The Csk model of Figure 7 is shown in its open conformation and without ATP. This rigid rotation of the upper domain of the catalytic core is typical for protein kinases. The Tyr-304 residue is located in the α -helix E, which runs on the surface of the lower lobe on the opposite side of the cleft forming the catalytic site (Figure 7). This residue is too far from the active site and substrate-binding residues to participate in catalysis.

There are only a few interactions between the upper and the lower lobe. In the closed conformation of cAPK there are six hydrogen bonds, two of which are present also in the open conformation (not shown). The interactions are mainly between the backbone oxygen and nitrogen atoms. According to our model, the sites conserved in the open conformation of cAPK are also present in Csk. One of the conserved interactions, the Tyr-304-Arg-244 interaction (corresponding to Tyr-156-Asn-99 in cAPK), requires side chain atoms and bridges helix E of the lower lobe with helix C of the upper lobe. The fact that this conserved hydrogen bonding is present in both the open and closed conformations of cAPK suggests a stabilizing effect on the integrity of the entire structure. The residue corresponding to Tyr-304 is almost invariant in protein kinases [21].

In conclusion, the analysis of the hydrogen bonding of the open and closed conformations reveals a net loss of several hydrogen bonds in the closed-to-open transition.

The Y304F mutation in Csk moderately but consistently reduced the activity of the enzyme. Notably, the Y304F protein was not inactive like the K222R mutant, the activity of which was undetected in poly-E/Y assays (Table 1) and using c-Src as a substrate [13]. Since pre-autophosphorylation of Csk (mainly on Tyr-304) did not affect its activity, the decrease in activity was presumably due to loss of the structure-stabilizing hydrogen bond making the molecule more flexible and therefore the

catalytic, closed, conformation more difficult to obtain. Conformational changes in protein kinases have to be finely tuned, and hydrogen bonds seem to play a critical role in these processes, as discussed above. The rotation of the upper domain is rigid and the extent of displacement depends on the particular protein kinase. While other protein kinases operate under different regulatory constraints, the putative Tyr-304-Arg-244 hydrogen bond appears to have a stabilizing effect on the active conformation of the kinase domain of Csk.

In conclusion, we show that Csk activity does not appear to be regulated by tyrosine phosphorylation, in contrast with most cytoplasmic PTKs. Instead, the low-stoichiometry phosphorylation of Tyr-184 might regulate localization of the kinase through protein-protein interactions in the appropriate cell compartment during certain stages of the cell cycle. Further, the high degree of conservation of Tyr-304 among protein kinases suggests that the corresponding residues in other kinases might have the same kinase domain-stabilizing function.

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