ELSEVIER

Il Farmaco 53 (1998) 266–272

IL FARMACO

Csk-mediated phosphorylation of substrates is regulated by substrate tyrosine phosphorylation [1]

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Abstract

Csk is a cellular protein tyrosine kinase (PTK) that has been shown to specifically regulate the activity of Src kinase family members by phosphorylation of a carboxy-terminal tyrosine residue. The molecular mechanisms controlling Csk regulation and its substrate specificity have not been elucidated. Here we report a novel type of overlay kinase assay that allows to probe for Csk-mediated phosphorylation of cellular substrates separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Most of the cell lines analyzed with this method revealed only a few potential Csk substrates. However, an increased number of Csk substrates was detected in NIH3T3 cells expressing a constitutively activated form of the Src kinase Lck or in PC12 and NIH3T3 cells that had been treated with pervanadate. These cells all display an increased level of cellular protein tyrosine phosphorylation which led to the conclusion that Csk preferentially phosphorylates tyrosine-phosphorylated proteins. To verify this hypothesis we analyzed Csk-mediated phosphorylation of recombinant Lck, a known Csk substrate. Results demonstrated that autophosphorylation of Lck (at Tyr394) facilitates Csk-mediated phosphorylation of Lck at its regulatory site (Tyr505). Subsequent peptide binding studies revealed that Csk can bind to a peptide corresponding to the Lck-autophosphorylation site only when it is phosphorylated. These findings suggest that autophosphorylation of Lck at Tyr394 triggers an interaction with Csk and thereby facilitates subsequent phosphorylation and inactivation of Lck. The phosphorylation of other cellular Csk substrates may be regulated by a similar mechanism.

Keywords: Lck; Src; Tyrosine kinase; Overlay kinase assay; Cellular signalling

1. Introduction

Csk is an ubiquitously expressed protein tyrosine kinase (PTK) which is structurally related to Src family kinases [1]. It displays SH2 and SH3 domains within the amino-terminal half of the protein; however, it lacks the autophosphorylation and the carboxy-terminal regulatory tyrosine residues which are conserved in all Src family kinases [1]. Furthermore amino-terminal myristylation, which has been shown to be crucial for targeting Src kinases to cellular membranes, is not observed for Csk [2]. Many reports have documented the importance of Csk in cellular development and growth control [3–5]. The generation of Csk-deficient mice revealed that Csk plays an important role for normal embryonic development, in particular for the development of the central nervous system.

Known Csk substrates include the members of the Src family of PTKs [2,6,7]. More importantly, Csk-mediated phosphorylation of Src kinases at their regulatory tyrosines has been demonstrated to down-regulate the corresponding kinase activities [8–12]. In addition, experiments in B and T cells demonstrated the importance of Csk-mediated regulation of Src family kinases such as Lyn and Lck. For example, Csk-negative chicken B-cell lines displayed hyperactive Lyn [13] and overexpression of Csk in a T-cell hybridoma cell line reduced the activation of Lck and production of IL-2 in response to antigen [5]. Both of these findings are consistent with and support the view that aberrant expression of Csk results in an associated inhibition of Src family kinases.

To fully understand the role of Csk in cellular regulation it will be crucial to define the factors that regulate the activity of Csk as well as the molecular mechanisms that control its substrate selection.

The identification of cellular substrates for a given kinase is a difficult task and factors that control substrate selection are difficult to ascertain. Recently, we have described an *Escherichia coli* expression system which allowed the purification of large amounts of soluble recombinant Csk protein [14]. This made it possible to develop an in vitro overlay kinase assay and to study at the molecular level Csk-mediated

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[1] Paper presented at the First Italian–Swiss Meeting on Medicinal Chemistry, Turin, Italy, September 1997.

0014-827X/98/$19.00
PII S0014-827X(98)00020-2

phosphorylation of cellular proteins, including the Src family kinase Lck. Comparative phosphorylation studies were used to demonstrate that tyrosine phosphorylated proteins, in particular autophosphorylated Lck, are preferentially phosphorylated by Csk. A model for a molecular mechanism by which Csk is targeted to its substrates is discussed.

2. Materials and methods

2.1. Cell cultures

NIH3T3 fibroblasts expressing mouse Lck (NIH3T3*lckwt*) or the constitutively activated form of Lck, LckF505 (NIH3T3*lckF505* [8]), were maintained in DMEM containing 10% fetal calf serum and Geneticin (400 μg/ml). Hut78 cells (ECACC# 88041901), derived from a human T-cell lymphoma, were maintained in RPMI 1640 (GIBCO/BRL) supplemented with 10% fetal calf serum, 1% penicillin, 1% streptomycin, and 2 mM glutamine. PC12 cells (ATCC# CRL 1721), a rat adrenal pheochromocytoma, were grown in DMEM containing 10% calf serum, 5% horse serum, 1% penicillin, 1% streptomycin and 2 mM glutamine. If necessary the cells were stimulated with 200 μM pervanadate at 37°C for 3 min [15] and then immediately washed with PBS. Cell lysates were prepared by resuspending 5×10^6 cells per ml in reducing SDS-sample buffer (50 mM Tris–HCl pH 6.8, 1.25 mM EDTA, 12.5% glycerol, 2.5 mM NaH_2P0_4, 0.025% Bromphenol Blue, 2% SDS, 10 mM DTT, 200 μM vanadate and 1.65% β-mercaptoethanol). Lysates were heated to 95°C for 5 min and chromosomal DNA was sheared by forcing the lysate 10 times through a 21 gauge needle. Lysates were stored frozen at −80°C.

2.2. Expression and purification of recombinant Lck and Csk

The NcoI-HindIII fragment of wild type human *lck* (*lckwt*) and two mutated forms of *lck* complementary DNA (*lckF394* and the *lckF394/F505* double mutant) were blunted with the Klenow fragment and cloned into the Hinc II site of the bacterial expression plasmid pDS56II, NcoI [16]. The resulting plasmids were transfected into M15 bacteria harboring the plasmid pREP4 [16]. To induce expression of Lck, bacteria were grown for 1 h in LB medium supplemented with IPTG (0.1 mM). The expression and purification of Csk and Lck has been described earlier [14,17,18].

2.3. Overlay kinase assay

Bacteria containing different forms of Lck and tissue culture cells were lysed in SDS-sample buffer, separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose filters. The nitrocellulose filters were pre-incubated for one hour in blocking buffer (0.15 M NaCl, 0.01 M $NaP0_4$ pH 7.2, 3% BSA and 0.2% Tween), washed in TN (0.15 M NaCl, 0.05 M Tris–HCl pH 7.2), and incubated for 20 min in kinase buffer (3 mM $MnCl_2$, 30 mM Hepes pH 6.8, 100 nM [γ-^{32}P]-ATP (1.5 Ci/mmol)) and 5 μg/ml purified Csk. The filters were then washed twice in 25 mM Tris base, 250 mM glycine and 0.1% SDS for 20 min and once in 25 mM Tris base, 250 mM glycine and 1% SDS for 20 min at 65°C. After drying, the nitrocellulose filters were analyzed by fluorography. All incubation and washing steps were performed at room temperature if not indicated otherwise.

2.4. Immunoblot analysis

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. Immunoblot analysis with either anti-phosphotyrosine rabbit antibodies or anti-Lck rabbit antiserum was performed as described earlier [9] using enhanced chemiluminescence as a detection method (ECL, Amersham, UK).

2.5. In vitro kinase assay

In vitro kinase assays were performed using purified recombinant Lckwt and LckF394 together with purified Csk. 100 ng of Csk were pre-incubated on ice in 20 μl of buffer A (30 mM Hepes pH 7.0, 3 mM $MnCl_2$) in the presence or absence of 10 ng of Lckwt or LckF394. The kinase reaction was started by addition of 10 μl of buffer B (30 mM Hepes pH 7.0, 3 mM $MnCl_2$, 50 nM [γ-^{32}P]-ATP (~200 Ci/mmol), 10 μg/ml BSA). After incubation at 4°C for 40 min the reaction was terminated by the addition of reducing SDS-sample buffer. Proteins were separated by SDS-PAGE and the dried gel was analyzed by fluorography and phosphoimager analysis.

2.6. Phosphopeptide mapping

Lck proteins from M15 cell lysates were phosphorylated on nitrocellulose as described above (overlay kinase assay). Purified recombinant proteins were phosphorylated in kinase assays as described above, separated by SDS-PAGE and transferred electrophoretically to nitrocellulose. Labeled proteins were located by autoradiography, excised from the filter and digested with trypsin [19]. Peptides were separated in two dimensions on cellulose thin-layer plates: (i) electrophoresis at pH 1.9 for 20 min, and (ii) ascending chromatography performed with 1-butanol/pyridine/acetic acid/water (75:50:15:60, vol./vol.) [20].

2.7. Peptide binding studies

Biotinylated peptides were synthesized as described earlier [9]. 10 μg of Y394 peptide (biotin-LARLIED-NEY$_{394}$TAREGAKFP) or P-Y394 peptide (biotin-LARLIEDNEpY$_{394}$TAREGAKFP) were incubated with 5 μg streptavidin-peroxidase complex (streptavidin–horserad-

ish-peroxidase, Pierce) in 50 μl blocking buffer (0.25% NP40, 3% BSA, TBS pH 7.4) for 15 min. Peptide bound to streptavidin-peroxidase was purified over a 2.5 ml G25 column equilibrated in blocking buffer. 1 μg aliquots of purified recombinant Csk and Lck were spotted onto nitrocellulose filters. The filters were blocked in blocking buffer for 1 h and then incubated for 30 min with peptide–streptavidin-peroxidase complexes. After washing the filters three times for 2 min with blocking buffer, bound peroxidase activity was assayed using enhanced chemiluminescence (ECL, Amersham, UK).

3. Results

3.1. Identification of Csk substrates using an overlay kinase assay

To identify potential substrates of Csk, cellular lysates were prepared from various different cell lines including Hut78 and AKR1 (both T-cell lines), PC12 (an adrenal pheochrome cytoma derived cell line), NIH3T3, NIH3T3*lckwt* and NIH3T3*lckF505* (all fibroblast derived cell lines). Similar amounts of total cellular protein were separated by SDS-PAGE and transferred onto nitrocellulose filters. Nitrocellulose filters blocked with BSA were incubated in kinase assay buffer in the presence of purified recombinant Csk and [γ-^{32}P]-ATP. Subsequently, the nitrocellulose filters were washed vigorously and phosphorylated proteins were detected by autoradiography (Fig. 1(a)). In the absence of Csk, no detectable incorporation of $^{32}P_i$ into membrane-bound proteins was observed (data not shown). Thus, we concluded that in this type of overlay kinase assay proteins bound to the nitrocellulose membrane are phosphorylated in a Csk-dependent manner.

The most prominent phosphorylated protein has an apparent molecular weight of about 55 kDa and was detected in all tested lysates (see Fig. 1(a)). In addition, several minor phosphorylated bands unique for a given cell line were revealed. Two closely related cell lines, NIH3T3 and NIH3T3*lckF505*, revealed the most striking difference in the Csk-mediated phosphorylation patterns. The NIH3T3*lckF505* cell line was derived from NIH3T3 cells by infection with recombinant retrovirus encoding LckF505, an activated form of the PTK Lck. As reported earlier, this results in cellular transformation and a 10-fold increase of cellular protein tyrosine phosphorylation [8]. Thus, in NIH3T3*lckF505* cells, elevated tyrosine phosphorylation of proteins might be responsible for the associated increase in Csk-mediated phosphorylation of cellular substrates.

To provide evidence for the importance of substrate tyrosine phosphorylation in Csk substrate selection, two different cell lines, NIH3T3 cells and PC12 cells, were stimulated with pervanadate. This treatment is known to increase dramatically cellular protein tyrosine phosphorylation levels within minutes (data not shown and Ref. [21]). Csk-mediated

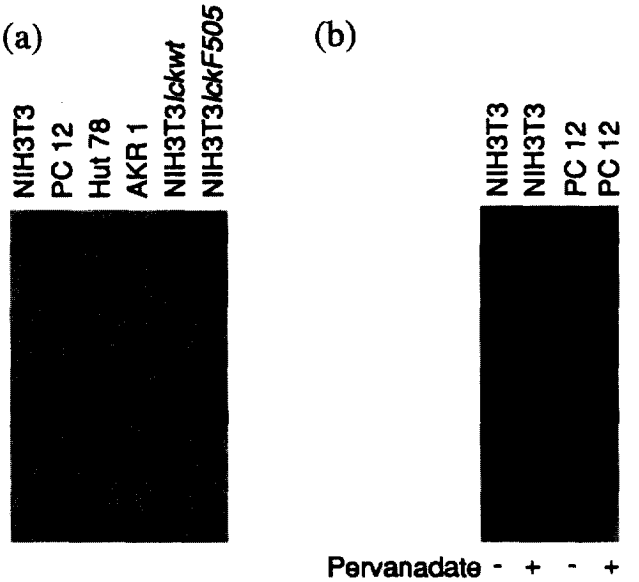

Fig. 1. *Overlay kinase assay.* (a) Lysates from the indicated cell lines were separated by SDS-PAGE and electroblotted onto nitrocellulose filters. The filters were incubated in kinase buffer together with purified recombinant Csk and [γ-^{32}P]-ATP. After intensive washing phosphorylated proteins were detected by fluorography. Note, the main substrate, an ~55 kDa band, is detected in all samples. Minor, unique substrates are detected in individual cell lines. (b) NIH3T3 or PC12 cells with (+) and without (−) stimulation with pervanadate (200 μM for 3 min) were lysed and analyzed by overlay kinase assay as described above. Note that treatment with pervanadate dramatically increased the number of apparent Csk substrates and cellular protein tyrosine phosphorylation (data not shown).

phosphorylation of proteins present in cellular lysates prepared before and after pervanadate treatment were compared using the overlay kinase assay technique described above. As shown in Fig. 1(b), pervanadate treatment resulted in a significant increase of Csk-mediated substrate phosphorylation. This suggests that substrate tyrosine phosphorylation is a critical factor in the substrate selection process of Csk.

3.2. Autophosphorylation of Lck facilitates Csk-mediated phosphorylation of the regulatory site of Lck

Csk can phosphorylate Lck specifically on Tyr505. This phosphorylation leads to a significant down-regulation of Lck kinase activity [2,14,22]. Here, we addressed the question of whether Lck autophosphorylation on Tyr394 influences Csk-mediated phosphorylation of Tyr505. In a first series of experiments we performed overlay kinase assays and probed three different forms of Lck (Lckwt, LckF394, and LckF394/F505) as substrates for Csk. Lckwt, when expressed in *E. coli*, has been shown to be preferentially phosphorylated on the autophosphorylation site Tyr394 [18]. LckF394 is a mutant form that lacks the autophosphorylation site and LckF394/F505 lacks both the autophosphorylation site and the regulatory tyrosine residue at the carboxy terminus. Bacterial strains expressing Lckwt, LckF394 or LckF394/F505 were solubilized in SDS-containing sample buffer and the resulting proteins were separated by SDS-PAGE followed by

transfer onto nitrocellulose filters. Three identical filters were produced. The first filter was analyzed by anti-Lck Western blot analysis and confirmed that similar amounts of Lck protein were applied to each lane (Fig. 2(a), top). The second filter was analyzed by antiphosphotyrosine Western blot analysis (Fig. 2(a), middle). Results demonstrated that only Lckwt, but not LckF394 or LckF394/F505, is significantly phosphorylated on tyrosine. The third nitrocellulose filter was probed with recombinant Csk using the overlay kinase assay. As shown in Fig. 2(a), bottom, the wt form of Lck is significantly more phosphorylated than the LckF394 mutant, and phosphorylation of LckF394/F505 is barely detectable. Two-dimensional peptide map analysis of phosphorylated Lckwt and LckF394 protein demonstrated that Csk-mediated phosphorylation of Lck proteins occurred on two major peptides (Fig. 2(b)); one contains Tyr505 and the other one an unknown phosphorylation site. This type of analysis confirmed that differences in Csk-mediated phosphorylation of LckF394 and Lckwt are due to differences in the phosphorylation of Tyr residue 505.

In the Csk overlay kinase assay described above the membrane-bound proteins were at least partially denatured. Thus, in a second series of experiments we tested whether Csk-mediated substrate phosphorylation of native proteins results in similar phosphorylation patterns. Native, recombinant Lckwt, LckF394 and Csk proteins were purified from *E. coli* expression systems and assayed under appropriate kinase conditions. Again Csk phosphorylated Lckwt (i.e. the autophosphorylated form of Lck) better than the unphosphorylated LckF394 mutant (Fig. 3(a)). Two-dimensional phosphopeptide map analysis of Lckwt and LckF394 (Fig. 3(b)) revealed that Csk-mediated phosphorylation of soluble protein was very similar to the phosphorylation of membrane-bound Lck proteins described above. To demonstrate obvious differences of Csk-mediated phosphorylation of Lckwt and LckF394 on the protein level it was important to minimize the apparent Lck autophosphorylation and to maximize for

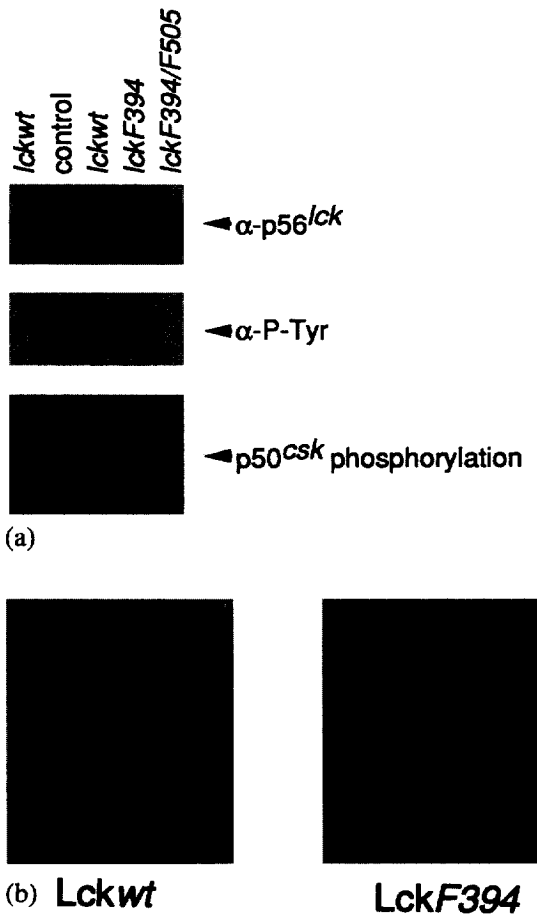

Fig. 2. (a) *Overlay kinase assay.* Bacteria expressing Lckwt, LckF394 or LckF394/F505 and control bacteria were lysed in sample buffer. Similar amounts of lysates or purified recombinant Lckwt (lane 1) were separated by SDS-PAGE and electroblotted onto nitrocellulose filters. Three identical blots were produced. The first was analyzed using anti-Lck antibodies; the second was analyzed using anti-P-Tyr antibodies and the third was analyzed by Csk overlay kinase assay. Note that all isoforms are equally expressed. However, only Lckwt is significantly tyrosine phosphorylated and can serve best as Csk substrate in the overlay kinase assay. (b) *Two-dimensional phosphopeptide map analysis.* The area of the nitrocellulose filter that contained in vitro phosphorylated Lckwt or LckF394 (Fig. 2(a)) was cut out, digested with trypsin and the released phosphopeptides were analyzed by two-dimensional thin-layer chromatography. The phosphopeptide containing Tyr505 is marked. Note that Tyr505 phosphorylation is significantly higher in Lckwt than in LckF394.

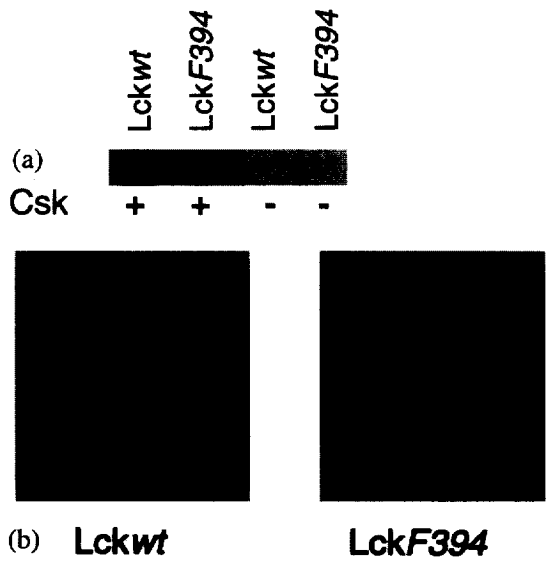

Fig. 3. (a) *In vitro kinase assay.* 10 ng of purified recombinant Lck (wt or LckF394) were incubated with (+) or without (−) 100 ng of purified recombinant Csk at 4°C in kinase buffer. After a 45 min incubation, samples were analyzed by SDS-PAGE and transferred to nitrocellulose filters. Note that under these assay conditions the observed phosphorylation was dependent on the presence of Csk and autophosphorylation of Lck was barely detectable. Fluorography and analysis with a phospho-imager revealed that the Csk-mediated incorporation into Lckwt is about 2.5 times higher than into LckF394 (data not shown). (b) *Two-dimensional phosphopeptide map analysis.* The area of the nitrocellulose filter containing in vitro phosphorylated Lckwt or LckF394 (Fig. 3(a)) was cut out, digested with trypsin and the released phosphopeptides were analyzed by two-dimensional thin-layer chromatography. The phosphopeptide containing Tyr505 is marked. Note that Tyr505 phosphorylation is significantly higher in Lckwt than in LckF394.

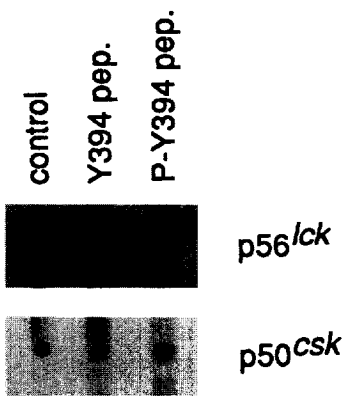

Fig. 4. *Peptide binding to purified Csk.* 1 μg of purified recombinant Csk or Lck was spotted onto nitrocellulose filters. The filters were then incubated with streptavidin-peroxidase complexes (control), with the biotinylated Y394 peptide (biotin-LARLIEDNEY$_{394}$TAREGAKFP) bound to streptavidin-peroxidase complexes or with biotinylated P-Y394 phosphopeptide bound to streptavidin-peroxidase complexes. The filters were washed, and bound peroxidase activity was detected using enhanced chemical luminescence (ECL, Amersham, UK). Note that Csk binds to the P-Y394 peptide but not to the unphosphorylated peptide.

Csk-mediated phosphorylation of Lck. This was achieved by reducing the reaction temperature and the relative amount of Lck. Using these optimized conditions, Csk-mediated phosporylation of Lck was prevailing. Taken together, the results of the in vitro kinase assays with the results of the previous overlay assay suggest that phosphorylation of Lck on Tyr394 facilitates subsequent Csk-mediated phosphorylation of Tyr505.

3.3. Csk binds to a phosphopeptide containing the Lck-autophosphorylation site Tyr394

On the molecular level, phosphorylated Tyr394 of Lck may provide a docking place for SH2-domain-containing Csk and facilitate the subsequent phosphorylation of the regulatory Tyr505. Thus, activated (i.e. autophosphorylated) Lck might provide the better Csk substrate than the nonphosphorylated Lck protein. To test this hypothesis we assayed the capability of Csk to bind to synthetic peptides corresponding to the phosphorylated or unphosphorylated Lck-autophosphorylation site (Y394 peptide and P-Y394 peptide). Specifically, we spotted purified recombinant Csk onto nitrocellulose filters and probed them with preformed complexes consisting of biotinylated peptide and streptavidin-peroxidase conjugates. Only the P-Y394 peptide containing the phosphorylated autophosphorylation site mediated the binding of peroxidase activity to Csk protein (Fig. 4). Control experiments using either the unphosphorylated peptide (Y394 peptide, Fig. 4) or a control phosphopeptide containing P-Tyr505 (data not shown) gave no signal above background. To further illustrate the specificity of the interaction of the P-Y394 peptide with Csk the same type of assay was performed with purified recombinant Lck. Neither the P-Y394 peptide nor the Y394 peptide displayed binding to Lck (Fig. 4), however it bound specifically and with much higher affinity a P-Y505-containing peptide (data not shown, and Ref. [9]).

4. Discussion

The identification of cellular substrates for a specific kinase is difficult to achieve and factors that control substrate selection processes by a particular PTK have been characterized only poorly at the molecular level. The availability of mg amounts of purified, recombinant Csk [14] allowed us to develop a new type of in vitro overlay kinase assay. This assay is suitable for the detection of potential Csk substrates present in complex mixtures of proteins. It combines the high resolution of SDS-PAGE analysis to separate proteins according to their size with the sensitivity of in vitro kinase assays in detecting potential substrates. When lysates of different cell lines were probed with purified Csk a major substrate, a protein of about 55 kDa, was detected in all of the cell lines tested. In addition, slightly different phosphorylation patterns were observed in individual cell lines. This may suggest that different cell types express common and different Csk substrates.

The finding that a protein can serve as a substrate in the overlay kinase does not prove but indicates that this protein is an in vivo substrate. In each case more elaborate studies will be needed to confirm that the observed in vitro phosphorylation is relevant in vivo. Nevertheless, this overlay kinase assay allows to probe quickly complex mixtures of proteins for potential protein kinase substrates. It will serve as a useful tool in the discovery of novel cellular substrates of specific kinases. Here we used this assay for studying at the molecular level factors that might regulate the substrate selection processes.

Despite numerous elegant and extensive biochemical and molecular studies on the catalytic mechanism of action of the Csk kinase [14], little is known about the regulation of Csk kinase activity and the factors that regulate its substrate selection. We have shown previously that purified recombinant Csk can undergo autophosphorylation on an unidentified tyrosine residue [14]. Such phosphorylation could theoretically affect Csk activity. However, up to now no post-translational modification could be linked to a change in Csk kinase activity [7]. On the other hand, using the overlay kinase assay technique we show now that an increase in the number of cellular proteins phosphorylated on tyrosine, induced either by expression of an activated form of Lck (Lck505) or treatment with pervanadate, results in an increase of the number of apparent Csk substrates. This observation suggests that tyrosine phosphorylation of cellular proteins triggers a specific interaction with Csk which results in subsequent, Csk-mediated, substrate phosphorylation. To provide direct evidence for such a mechanism we analyzed in more detail the phosphorylation of a well-defined Csk substrate: the Src family kinase Lck. Specifically, we tested the potential of wt and mutant forms of recombinant Lck

expressed in *E. coli* to serve as substrates for Csk. Two different types of assays, the overlay kinase assay and a standard in vitro kinase assay, both revealed that autophosphorylation of Lck on Tyr394 facilitates Csk-mediated phosphorylation on Tyr505. Two-dimensional peptide map analysis confirmed that the difference in Csk-mediated Lck phosphorylation correlates with a difference in phosphorylation of the regulatory site Tyr505, a site known to be phosphorylated by Csk in vivo as well.

Binding studies revealed that a peptide representing the autophosphorylation site of Lck binds to Csk in a phosphorylation-dependent manner. Most likely, this interaction is mediated by the SH2 domain of Csk. This view is supported by an in vitro study analyzing the binding specificity of the Csk-SH2 domain using peptide libraries [23]. It was postulated that the SH2 domain of Csk can interact with phosphorylated tyrosine residues in the autophosphorylation site of Src kinases. This observation, together with the results of our biochemical analysis, suggests that activation (i.e. autophosphorylation) of Lck at Tyr394 creates a docking site for Csk. Directed binding of Csk to Lck thereby may facilitate subsequent Csk-mediated phosphorylation of the regulatory site Tyr505 of Lck. The differences in Csk-mediated phosphorylation of Tyr505 observed between autophosphorylated Lckwt and the LckF394, albeit low (2–3-fold), support the view that Csk-mediated phosphorylations are controlled on the level of protein–protein interactions. This model is strongly supported by two recent in vivo studies analyzing the functional role of Csk in T-cell activation. The first study [5] indicated that the extent of C-terminal tyrosine phosphorylation of Lck in resting T cells is not changed by Csk overexpression; however, TCR-mediated activation of these cells resulted in reduced activation of Src family kinases. It was suggested that only the fraction of Src-related molecules which participates in a given signaling response (i.e. the fraction which is autophosphorylated) is actively down-regulated by Csk. These observations are consistent with our finding that Csk preferentially phosphorylates autophosphorylated Lck. The second study [24] provided evidence that the SH2 domain of Csk is crucial for Csk-mediated phosphorylation of Tyr505 and down-regulation of Lck activity. This, together with our results, suggests that an interaction of Csk with phosphorylated Lck may involve the SH2 domain of Csk and phosphorylated Tyr394 of Lck. Such an interaction may facilitate Csk-mediated phosphorylation of Tyr505 in vitro and in vivo. Of course, this mechanism would only explain the appearance of doubly phosphorylated Lck molecules upon T-cell activation. To allow the generation of inactive, Y505-phosphorylated Lck at least one PTPase has to be involved.

5. Nomenclature

ATP	adenosine 5′-triphosphate
BSA	bis(trimethylsilyl)acetamide
DMEM	Dulbecco's modified Eagle medium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani (GIBCO/BRL)
NP40	Nonidet P40
PBS	phosphate buffered saline
PTK	protein tyrosine kinase
PAGE	polyacrylamide gel electrophoresis
PTPase	protein tyrosine phosphatase
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline (25 mM Tris–HCl, 0.8% NaCl, 0.02% KCl, pH 7.4)

Acknowledgements

We would like to thank Dr Martin Stieger for constructing the plasmids necessary for bacterial expression of Csk and Dr Jarema Kochan for critical reading of the manuscript.

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