Rapid and efficient purification of Src homology 2 domain-containing proteins: Fyn, Csk and phosphatidylinositol 3-kinase p85

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To analyse the regulation of Src family tyrosine kinases *in vitro*, we have purified Fyn and Csk, a kinase capable of regulating Fyn activity by phosphorylation, from baculovirus-infected insect cells. The proteins were purified by affinity purification over a phosphotyrosine column. Highly purified proteins were eluted from the resin by a salt gradient and further purified by ion-exchange chromatography. This purification scheme was successfully applied to a third, unrelated protein that also contains the Src homology 2 (SH2) domain, namely the 85 kDa subunit of phosphatidylinositol 3-kinase, indicating that this method is versatile and should prove applicable to any protein with an accessible SH2 domain. The binding of Csk to different phosphopeptides was tested, and specificity for the autophosphorylation site of Fyn was demonstrated. Pure Csk was used to phosphorylate Fyn and down-regulate its kinase activity, and the kinetic parameters of both the active and the repressed forms of Fyn were determined. Repression of Fyn activity by Csk reduced binding of Fyn to phosphopeptides to undetectable levels, supporting the model that predicts an intramolecular interaction of the Fyn SH2 domain with a C-terminal phosphotyrosine residue.

INTRODUCTION

The Src family of tyrosine kinases is a group of nine membrane-anchored kinases lacking transmembrane domains (reviewed in Bolen, 1993; Courtneidge, 1994). Even though their precise function remains unclear, they all seem to be involved in some aspects of signal transduction. Where tested, deregulation of their kinase activity leads to oncogenic transformation of cells, stressing that they are positioned at key control points of cellular growth (Levinson et al., 1978; Marth et al., 1986; Nishizawa et al., 1986; Voronova and Sefton, 1986; Sukegawa et al., 1987; Semba et al., 1990). Indeed, many members of the Src family have been shown to bind to transmembrane receptor molecules after ligand binding or antibody cross-linking of receptor, and in many cases binding is accompanied by an increase in their kinase activity (reviewed in Cantley et al., 1991; Bolen et al., 1992). Other potential functions of Src family kinases include a role during mitosis (reviewed in Taylor and Shalloway, 1993), as well as perhaps roles in more specialized processes such as secretion and bone resorption by osteoclasts (Parsons and Creutz, 1986; Horne et al., 1992; Kaplan et al., 1992; Lowe et al., 1993).

Src family tyrosine kinases share a similar organization, consisting of five subdomains (reviewed in Koegl and Courtneidge, 1992). In brief, the first 15 amino acids provide signals for the attachment of one or more fatty acids (either myristate alone, or both myristate and palmitate), which contribute to membrane localization (Resh, 1990; Paige et al., 1993; Shenoy-Scarcia et al., 1993) (M. Koegl, P. Zlatkine, S. Ley, S. A. Courtneidge and A. I. Magee, unpublished work). The adjacent 40–70 amino acids make up the region of greatest divergence within the family, and is termed the unique domain. Next come two domains that have been implicated in protein–protein interactions, the Src homology 3 (SH3) and SH2 domains. SH2 domains recognize and bind to phosphotyrosine within a defined environment of amino acid sequences (which provide the specificity of the interaction), whereas SH3 domains bind to short sequences rich in proline (reviewed in Pawson and Schlessinger, 1993). The C-terminal half consists of the catalytic domain followed by a short tail of ~ 20 amino acids containing Tyr-527 (numbered according to the sequence of chicken c-*src* gene product, cSrc), an important regulatory phosphorylation site (Cooper et al., 1986; Cooper and Howell, 1993). Repression is most probably brought about by the binding of the SH2 domain of cSrc to its own phosphorylated tail, resulting in a conformation unfavourable for enzymic activity (Matsuda et al., 1990; Roussel et al., 1991; Murphy et al., 1993; Okada et al., 1993; Superti-Furga et al., 1993). Dephosphorylation of this site increases the $V_{max.}$ of cSrc 10-fold (Courtneidge, 1985).

Recently, a kinase that can phosphorylate a number of Src family tyrosine kinases at this site both *in vitro* and *in vivo* has been purified and cloned (Okada and Nakagawa, 1989; Nada et al., 1991; Partanen et al., 1991; Bräuninger et al., 1992). This enzyme, called Csk, shows a similar organization to the Src family tyrosine kinases. It also has an SH3 and an SH2 domain N-terminal to the kinase domain, but lacks the unique domain, the acylation domain and the regulatory phosphorylation site in the tail. Mice lacking a functional gene for Csk die during embryogenesis from severe defects, including faulty neural tube formation (Imamoto and Soriano, 1993; Nada et al., 1993). The specific activities of several Src family tyrosine kinases isolated from these mice are increased, indicating that Csk is crucial in their regulation.

In the present paper we describe the expression and purification of both Fyn (a Src family tyrosine kinase) and Csk from insect cells expressing these proteins via infection with baculovirus vectors. Purification over a phosphotyrosine resin proved to be a fast and efficient means of purification of not only Fyn and Csk but also another unrelated SH2 domain-bearing protein. The

Abbreviations used: DTT, dithiothreitol; NP40, Nonidet P40; PDGF, platelet-derived growth factor; SH2, Src homology 2; SH3, Src homology 3.

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production of pure kinases allowed us to assess the effects of phosphorylation of Fyn at its C-terminal regulatory site (Tyr-531) on the activity of the enzyme and on the availability of its SH2 domain for binding to relevant tyrosine-phosphorylated peptides.

MATERIAL AND METHODS

Materials

Actigel was from Sterogene, CNBr-activated Sepharose, MonoQ and MonoS columns were from Pharmacia. [γ-^{32}P]ATP (> 5000 Ci/mmol) was from Amersham. Protein molecular mass markers were from BioRad. Sodium orthovanadate was from Aldrich. All other chemicals were from Sigma.

Generation of baculoviruses expressing Csk, Fyn, and Fyn^{G2A,K299M}

To clone the human Csk cDNA into the vector pACMY1, it was first cloned into the vector pSP73 as a *Sac*I fragment, such that the 5′ end of the gene was next to the *Kpn*I site of the vector. Next, it was cut out of pSP73 via the *Cla*I and *Hin*dIII sites and ligated into the corresponding sites of the vector pCPXS. From there, it could be cut out as a *Bam*HI fragment and ligated into the *Bam*HI site of pAcMY1. Wild-type Fyn and a mutant lacking the myristylation sequence (Gly-2 mutated to alanine) and kinase activity (Lys-299 mutated to methionine), termed Fyn^{G2A,K299M}, were inserted into the baculovirus transfer vector pACC4 (Twamley et al., 1992). Passage and transfer of insect cells, selection and growth of plaques and virus infections were carried out as described by Summers and Smith (1987).

Purification of Csk from insect cells

Sf9 cells (*Spodoptera frugiperda* cell line) were grown in 150 ml cultures in 1.0 l spinner flasks to a density of 2–3 × 10^6 cells/ml, pelleted by centrifugation at 100 *g* and resuspended in 30 ml of virus-containing medium from previous infections. After 1 h, fresh medium was added to a cell density of 2 × 10^6 cells/ml. After 62 h of infection, cells were harvested by centrifugation at 100 *g*, washed once in PBS, and lysed in 0.7 ml per 1 × 10^7 cells of ice-cold buffer A [0.1 % (v/v) Nonidet P40 (NP40), 20 mM Hepes, pH 7.5, 2 mM dithiothreitol (DTT), 10 mM NaF, 100 μM sodium orthovanadate (made up as described in Kypta et al., 1988) 20 μM leupeptin, 1 % (v/v) aprotinin and 100 μM phenylmethanesulphonyl fluoride (PMSF)]. The entire procedure was carried out at 4 °C. Lysates were cleared by centrifugation at 35000 *g* for 30 min. The supernatant was filtered through a 45 μm-pore-size filter and loaded on to a poly(Glu,Tyr)column, comprising a synthetic random polymer of poly(Glu·Tyr, 4:1), (bed volume 7ml, see below) at 1 ml/min. Csk was eluted with a gradient of NaCl (0–400 mM, gradient volume 35 ml). At all purification steps, fractions were analysed for the presence of Csk by SDS/PAGE and Coomassie Blue staining. Positive fractions were pooled, diluted with 4 vol. of buffer A′, loaded on a MonoQ column and eluted with a gradient of NaCl (0–500 mM, gradient volume 30 ml). Positive fractions were pooled and diluted with 9 vol. of buffer A (buffer A, pH 7), loaded on a MonoS column and eluted with a gradient of NaCl (0–500 mM, gradient volume 30 ml). Positive fractions were pooled, dialysed against 20 mM Hepes, pH 7.5, 2 mM DTT and stored in liquid nitrogen. Repeated freeze-thawing (up to 10 times) in liquid nitrogen did not affect the kinase activity of the protein.

As an alternative method of purification, lysates prepared and clarified as described above were loaded on a column containing phosphotyrosine coupled to Actigel (bed volume 5.5 ml, see below) at 0.5 ml/min and eluted with a gradient of NaCl (0–1000 mM, gradient volume 30 ml). Positive fractions were pooled, diluted 10-fold with buffer A′ and passed over a MonoS column as described above.

Purification of Fyn from insect cells

For large-scale infection of insect cells with Fyn-expressing virus, cells were grown in spinner flasks to a density of 2–3 × 10^6 cells/ml and seeded on to 150 cm^2 dishes (2 × 10^7 cells/dish) for infection. Infection in spinner flasks did not lead to reproducible yields, probably because cells were more sensitive to shearing forces due to cytotoxic effects of the large increase in tyrosine kinase activity. Cells were infected with 2 ml of virus in 20 ml of fresh medium per 150 cm^2 dish for 62 h. Cells were detached by gently knocking the dish against a stable support, pelleted, washed, lysed and cleared as described for Csk with the exception that buffer A contained 1 % NP40. Lysates were then loaded on to a column containing phosphotyrosine coupled to Actigel (bed volume 5.5 ml, see below) at 0.5 ml/min, washed with buffer A and eluted with a gradient of NaCl (100–700 mM, gradient volume 25 ml) at 0.5 ml/min. At all purification steps, fractions were analysed for the presence of Fyn by SDS/PAGE and Coomassie Blue staining. Positive fractions were pooled, diluted with 9 vol. of 10 mM Tris, pH 7.5, 1 mM DTT and loaded onto a MonoQ column. Protein was eluted with a gradient of NaCl (0–500 mM, 12 ml). Positive fractions were pooled, dialysed against 1.0 l of 10 mM Tris, pH 7.5, 0.1 % NP40 and 2 mM DTT and stored in liquid nitrogen. Fyn^{G2A,K299M} was purified after exactly the same protocol with the exception that buffer A contained 0.1 % NP40 instead of 1 % NP40. In some of the early preparations of Fyn an additional poly(Glu,Tyr) column was used between the phosphotyrosine column and the MonoQ column. However, this step turned out to be unnecessary for efficient purification.

Purification of p85 from insect cells

After a 72 h infection of an adherent culture (as described for Fyn), cells were removed from the dish, washed with PBS and resuspended in 0.7 ml buffer C (buffer A without NP40)/10^7 cells. Cells were broken in a dounce homogenizer with 20 strokes and the suspension was clarified by centrifugation at 35000 *g* for 30 min. The extract was then passed through a 45 μm filter, loaded on to a phosphotyrosine column and eluted with a gradient of NaCl in buffer C (0–1000 mM, gradient volume 30 ml). Fractions containing the pure protein were dialysed against a buffer of 20 mM Tris, pH 7.5, 2 mM DTT and concentrated in a Centricon 30 microconcentrator (Amicon).

Preparation of affinity resins

Poly(Glu,Tyr) was coupled to CNBr-activated Sepharose as described for coupling of peptide ligands for affinity purification of antibodies (Kypta et al., 1988). Phosphotyrosine and phosphopeptides were coupled to Actigel ALD Superflow (Sterogene) according to the manufacturer's instructions. The coupling concentrations of phosphotyrosine and phosphopeptides were 10 mg/ml and 500 μM respectively. Both resins were stored at 4 °C in 20 % (v/v) ethanol and remained functional for at least 1 year.

Assay of protein kinase activity

Tyrosine kinase activity was assayed in 20 mM Hepes, pH 7.5, 1 mM DTT, 10 mM $MnCl_2$, 0.1% NP40 and [γ-^{32}P]ATP using poly(Glu,Tyr) (100 μg/ml), heat- and acid-denatured enolase (0.25 mg/ml) or pure $Fyn^{G2A,K299M}$ (250 nM) as a substrate. Total reaction volume was 20 μl. After 4 min, reactions were terminated by addition of 4 x Laemmli sample buffer and boiling for 1 min. Reaction products were analysed on a 9% (w/v) polyacrylamide gel and autoradiography or processed on a phosphorimager (Molecular Dynamics).

Binding of proteins to phosphopeptides

The sequences of the phosphopeptides used in this study are: 416, IEDNEYTARQGA; 527, ATEPQYQPGENL; 857, DIMRDSNYISKGST; and 579, IESVSSDGHEYIYVDP; whereby the underlined residue represents the phosphorylated tyrosine. Coupling of phosphopeptides to Actigel was performed according to the manufacturer's instructions at a concentration of 500 μM peptide in Hepes buffer (pH 7.5). For binding assays, the peptide-coupled beads were diluted 1:5 with non-coupled beads. Binding of pure proteins was performed for 1 h at 4 °C in 150 mM NaCl, 1% NP40, 20 mM Tris, pH 7.5 in a volume of 100–200μl for 10 μl of beads on a rotating wheel. Unbound material was removed by washing the beads three times with 500 μl of the same buffer and once with 500 μl 20 mM Tris, 150 mM NaCl. Bound proteins were eluted by boiling in Laemmli sample buffer and analysed by SDS-PAGE and Western blotting as described by Twamley et al. (1992), employing detection by enhanced chemoluminescence (Amersham).

Antibodies

Antibodies used in this study were the antibody cst.1, which recognizes cSrc, Fyn and cYes, the anti-[platelet-derived growth factor (PDGF) receptor] antibody PR4 and the anti-Csk antibody Csk2. All are rabbit polyclonal antibodies and have been described before (Kypta et al., 1990) except Csk2, which was raised using Csk purified from insect cells as an antigen.

RESULTS

Expression of tyrosine kinases in insect cells

To generate large amounts of Fyn and Csk proteins and analyse their biochemical properties, we sought to express Fyn as well as Csk in baculovirus-infected insect cells. The coding regions of the human Fyn and Csk cDNAs were cloned into baculovirus expression vectors under the control of the strong polyhedrin promoter. The generation of baculoviruses able to express Fyn has been previously described (Twamley et al., 1992). Recombinant viruses were obtained by *in vivo* recombination with wild-type baculovirus and screening for polyhedrin-negative plaques. Infection of Sf9 cells with a baculovirus engineered to express Csk led to the accumulation of Csk protein to levels as high as 20% of total cell protein. Expression of Csk had no obvious effects on the viability of infected cells within the first 62 h of infection. In contrast, expression of wild-type Fyn protein appeared to have toxic effects in insect cells. When infections were performed in spinner flasks, about half of the cells were already lysed after 40 h, probably due to their higher susceptibility to mechanical stress. This toxicity effect may have been brought about by the high kinase activity of Fyn, or because it is a membrane-associated protein, since a kinase-inactive, cytoplasmic version of Fyn (with a mutated ATP-binding site and myristylation sequence, $Fyn^{G2A,K299M}$) had no such effect on the cells. However, expression of this protein also did not reach levels comparable with those of Csk (compare Figures 1a and 5a, lanes S).

Purification of Fyn and Csk from insect cells

To purify Fyn and $Fyn^{G2A,K299M}$, we made use of the affinity of the Fyn SH2 domain for phosphorylated tyrosine. Although the natural binding site for an SH2 domain consists of a short stretch of amino acids surrounding a phosphorylated tyrosine, with the identity of the surrounding residues being important for high-affinity binding (Fantl et al., 1992; Songyang et al., 1993), the domain also displays a low affinity for isolated phosphotyrosine (Mayer et al., 1991). An affinity resin was prepared by coupling phosphotyrosine to Actigel. Fyn bound efficiently to this resin and could be eluted with an NaCl gradient as the only major band on Coomassie-Blue-stained gel (Figure 1a). Fyn eluted from the gradient between 200 and 600 mM NaCl. Due to this quick and efficient first step, contamination by breakdown products of Fyn, which has been a notorious problem in the purification of Src family tyrosine kinases (Varshney et al., 1986; Presek et al., 1988; Radziejewski et al., 1989; Feder and Bishop, 1990), could be kept to a minimum. Further purification and concentration was achieved by purification over a MonoQ column (Figure 1b). By this method, 5 μg of active Fyn and 1 mg of $Fyn^{G2A,K299M}$ could be obtained from 5×10^8 cells. We estimated that the proteins were more than 95% pure.

We originally purified Csk using a simplified protocol based on the method described by Nada et al. (1991), which involved purification by chromatography on a poly(Glu,Tyr) resin and subsequent chromatography on MonoQ and MonoS columns. This method resulted in a yield of ~5 mg of >95% pure protein from 5×10^8 cells (results not shown). In later prepara-

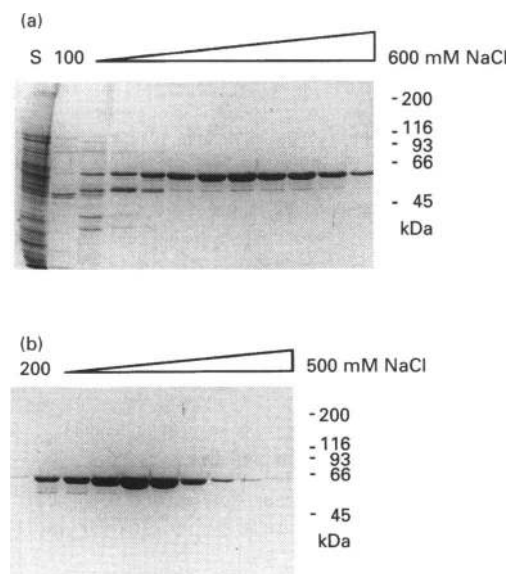
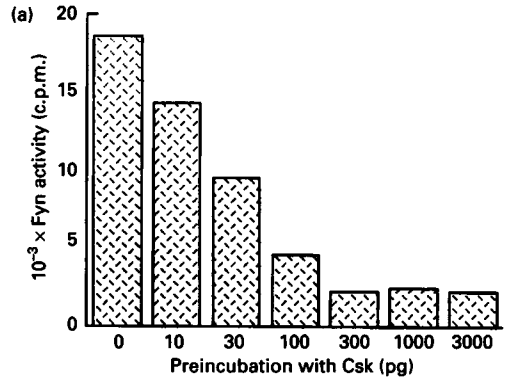
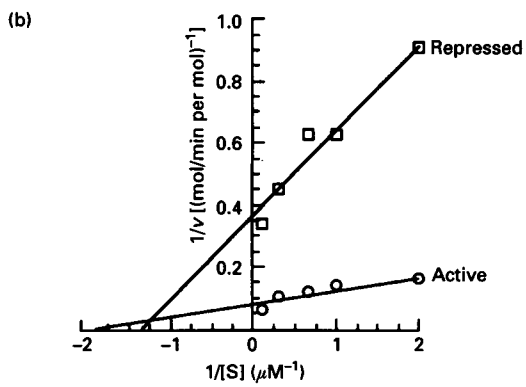


Figure 1 Purification of $Fyn^{G2A,K299M}$ from Sf9-cells

The different fractions were analysed on an SDS/9% (w/v) polyacrylamide gel and proteins were visualized by Coomassie Blue staining. (**a**) Purification over a phosphotyrosine column. Lane S, starting material. (**b**) Purification over a MonoQ column. Bound proteins were eluted with a gradient of NaCl as indicated in the Figure. Molecular-mass marker positions are indicated on the right.





(c)

Fyn	Active	Repressed
K_m (μM)	1.1 ± 0.4	0·8 ± 0.2
$V_{max.}$ (mol/min per mol)	11.1 ± 1.1	2.3 ± 0·6

Figure 2 Regulation of Fyn tyrosine kinase by Csk *in vitro*

(**a**) *In vitro* kinase assay of Fyn using denatured enolase as a substrate. Fyn (250 pg) was incubated without Csk or in the presence of the indicated amounts of Csk before adding denatured enolase and [γ-^{32}P]ATP. As our preparations of Csk do not phosphorylate enolase, this assay measures only Fyn activity. The reaction products were separated on a 9% (w/v) polyacrylamide gel and analysed on a phosphoimager (Molecular Dynamics). (**b**) Lineweaver–Burk plot of the activity of the two forms of Fyn. To generate Fyn in its repressed form, 5 ng of Fyn was incubated with or without 90 ng of Csk and ATP for 10 min at 30 °C. Phosphorylated or non-phosphorylated Fyn (250 pg) was then taken to determine its specific kinase activity as described in (**a**). (**c**) Kinetic parameters of Fyn as determined in (**b**).

tions, Csk was purified over a phosphotyrosine resin followed by a MonoS column (see below).

Kinetic properties of purified Fyn and Csk

The turnover number of Csk was determined using two known substrates of the enzyme, namely poly(Glu,Tyr) and purified Fyn^{G2A,K299M}. Using 5 μM ATP and a substrate concentration of 0.1 μg/μl for poly(Glu,Tyr) and 250 nM for Fyn^{G2A,K299M}, the turnover numbers were 4.6 and 4.8 mol/min per mol of Csk respectively. Previous purifications of Csk from neonatal rat brain and *Escherichia coli* have given similar values of 5.25 and 3.15 mol/min per mol (Okada and Nakagawa, 1989; Bougeret et al., 1993). In contrast with reports using Csk purified from *E. coli*, our preparations did not display any activity towards enolase or autophosphorylation activity when we tested enzyme concentrations up to 3 nM. Possibly the fact that Bougeret et al. (1993) used enzyme concentrations two orders of magnitude higher than those that we employed in our experiments accounts for the difference. We have not tested enzyme concentrations this high because it would not be possible to exclude the contribution of minor contaminating insect cell tyrosine kinases.

The tyrosine kinase activity of Fyn was determined using heat- and acid-denatured enolase as a substrate. The $V_{max.}$ of the enzyme was 11.1 mol/min per mol. This value obtains to the form of Fyn which was not phosphorylated at Tyr-531, for which our purification method is selective (see below). This is within the range of $V_{max.}$ values reported for the closely related kinase cSrc, which is 39 mol/min per mol for cSrc^{G2A} purified from insect cells (Lydon et al., 1992) and 1.5 mol/min per mol for cSrc purified from human platelets (Feder and Bishop, 1990). To study the effects of phosphorylation of Fyn by Csk on its activity, we incubated pure Fyn with increasing amounts of Csk in the presence of ATP before adding enolase and radioactive ATP. Since enolase was not a substrate for our preparations of Csk, its phosphorylation reflects only the activity of Fyn. As depicted in Figure 2(a), phosphorylation of Fyn by Csk resulted in a repression of Fyn kinase activity. This repressed form of Fyn exhibited a 5-fold reduction in $V_{max.}$, but the K_m for ATP was only minimally changed (Figures 2b and 2c). Thus, phosphorylation of the C-terminal tail of Fyn repressed its enzymic activity by reducing $V_{max.}$ This is in agreement with the observed regulation of the closely related kinase cSrc by C-terminal phosphorylation (Courtneidge, 1985; Okada and Nakagawa, 1989).

Csk binds to a phosphopeptide modelled on the autophosphorylation site of cSrc

Like Src family tyrosine kinases, Csk has one SH3 and one SH2 domain N-terminal to its kinase domain. However, the binding specificities of these domains are not known. We tested whether Csk could bind to either of the two tyrosine phosphorylation sites of its substrate cSrc. To this end, two phosphopeptides were synthesized corresponding to the regions surrounding Tyr-416 (the autophosphorylation site) and Tyr-527 of cSrc. Due to the high degree of similarity among Src family kinases, the sequence of the 416 phosphopeptide is identical with the sequence of the autophosphorylation site in Fyn, and the 527 phosphopeptide only differs by a single amino acid. After coupling of the phosphopeptides to Actigel beads, the beads were incubated with Csk protein and washed extensively and the bound material was analysed by immunoblotting. Csk bound detectably to the peptide (416) corresponding to the autophosphorylation site of cSrc (Figure 3). No binding was observed when the non-phosphorylated version of this peptide was used. This binding appeared to be specific, because Csk did not bind to either the 527 phosphopeptide or to a phosphopeptide modelled on the autophosphorylation site of the PDGF receptor (Figure 3). The integrity of the 527 phosphopeptide was verified by its ability to bind the Fyn SH2 domain (results not shown). Furthermore, the binding assay was conducted using salt concentrations in which Csk did not bind efficiently to phosphotyrosine itself, further indicating that this interaction was specific.

Effect of phosphorylation by Csk on the accessibility of the SH2 domain of Fyn

A model has been proposed which suggests that the tail phosphorylation of Src family tyrosine kinases triggers an intra-

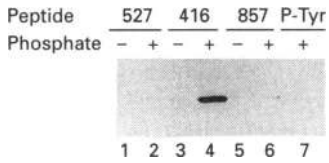


Figure 3 Csk binds a peptide modelled on the autophosphorylation site of Src family tyrosine kinases

Pure Csk was incubated with beads covalently coupled to non-phosphorylated peptides (−) or tyrosine-phosphorylated peptides (+) modelled on the autophosphorylation sequence of Fyn (416), the C-terminal tyrosine phosphorylation site of cSrc (527) or the sequence surrounding Tyr-857 of the PDGF receptor (857). After extensive washing, the bound material was released by boiling in Laemmli sample buffer and analysed by SDS/PAGE followed by Western blotting using the αCsk antibody Csk2. P-Tyr, phosphotyrosine (control).

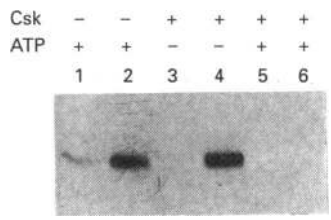


Figure 4 The SH2 domain of Fyn is blocked for binding to tyrosine-phosphorylated peptides after phosphorylation by Csk

$Fyn^{G2A,K299M}$ (100 ng) was preincubated with 25 μM ATP alone (lanes 1 and 2), Csk (1 μg) alone (lanes 2 and 3) or Csk (1 μg) and 25 μM ATP for 10 min at 30 °C. Thereafter, it was incubated with beads covalently coupled to non-phosphorylated peptides (odd lanes) or tyrosine-phosphorylated peptides (even lanes) modelled on the binding site for Src family tyrosine kinase of the PDGF receptor (Tyr-579) at 4 °C. After extensive washes, the remaining material was released by boiling in Laemmli sample buffer and analysed by SDS/PAGE and Western blotting using the antibody cst.1.

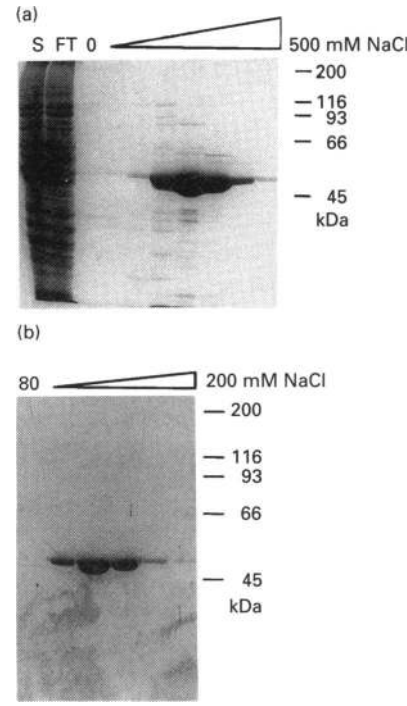


Figure 5 Purification of Csk over phosphotyrosine and MonoS columns

The different fractions were analysed on an SDS/9% (w/v) polyacrylamide gel and proteins were visualized by Coomassie Blue staining. (**a**) Purification over a phosphotyrosine column. Lane S, starting material; lane FT, flow-through. (**b**) Purification over a MonoS column. Bound proteins were eluted with a gradient of NaCl as indicated in the Figure. Molecular-mass marker positions are indicated on the right.

molecular association of the SH2 domain with the phosphotyrosine at the C-terminus, resulting in an inactive conformation (Matsuda *et al.*, 1990; Cantley *et al.*, 1991; Koch et al., 1991). Support for this idea comes from the fact that some mutations in the SH2 domain of Src can release repression via C-terminal phosphorylation (Hirai and Varmus, 1990; O'Brien et al., 1990; Seidel-Dugan et al., 1992). Furthermore, an activated version of Src which lacks Tyr-527 can bind to a peptide modelled on the sequences surrounding Tyr-527, but repressed cSrc or activated cSrc with a mutated SH2 domain cannot (Roussel et al., 1991; Cobb and Parsons, 1993). Finally, using heterologous expression systems, it has been shown that Csk can only regulate Src when the SH2 domain of Src is intact (Murphy et al., 1993; Okada et al., 1993; Superti-Furga et al., 1993). Since Src family tyrosine kinases are highly homologous with one another, this model, if correct, should apply to all members of this class. One important prediction is that not only a mutation at Tyr-531 (in the case of Fyn) but also a change in the phosphorylation state at this site should affect the accessibility of the SH2 domain for binding of other tyrosine-phosphorylated amino acid sequences. The ability to generate pure Fyn with and without the phosphate at Tyr-531 enabled us to test this prediction *in vitro*.

A phosphopeptide modelled on the binding site of Src family tyrosine kinases on the PDGF receptor (Mori et al., 1993) was coupled to Actigel beads to function as a binding site for the Fyn SH2 domain. To test the binding of the two forms of Fyn (phosphorylated or non-phosphorylated at the tail) to the peptide, $Fyn^{G2A,K299M}$ was incubated in the presence of ATP with or without saturating amounts of Csk. The use of kinase-inactive Fyn in this experiment allowed us to exclude any effects of the kinase activity of Fyn. The proteins were allowed to bind to the phosphopeptide resin and unbound proteins were removed by washing. After separation by SDS/PAGE the fraction of Fyn that bound to the peptide was visualized by immunoblotting. As shown in Figure 4, non-phosphorylated Fyn bound efficiently to the phosphopeptide, but Csk-phosphorylated Fyn showed greatly reduced binding, thus fulfilling the prediction of the model. This inhibition only occurred when ATP was included in the kinase reaction mixture, indicating that phosphorylation was required and that the inhibition was not brought about by competition of the SH2 domain of Csk for the binding site.

Purification of SH2 domain containing proteins by affinity purification over a phosphotyrosine column

A large number of proteins have SH2 domains, which can bind to different amino acid sequences surrounding the phosphorylated tyrosine (Pawson and Schlessinger, 1993). Since all of these proteins share a basic affinity for phosphotyrosine, it should be possible to purify any protein containing this domain on a phosphotyrosine resin. We tested this idea for two further proteins. As a second tyrosine kinase, we tested whether Csk could also be purified in this manner. As depicted in Figure 5(a), the protein bound to the resin and could be eluted by a salt gradient. In contrast to Fyn, Csk eluted early in the gradient at

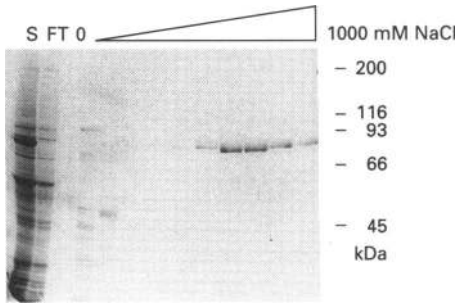


Figure 6 Purification of p85 over a phosphotyrosine column

The different fractions were analysed on an SDS/7.5% (w/v) polyacrylamide gel and proteins were visualized by Coomassie Blue staining. Lane S, starting material; lane FT, flow-through. Bound proteins were eluted with a gradient of NaCl as indicated in the Figure. Molecular-mass marker positions are indicated on the right.

Table 1 Purification of different SH2-domain-containing proteins over phosphotyrosine

Expression levels and percentage of binding to phosphotyrosine were determined by Coomassie Blue staining after PAGE. Expression levels are calculated as the percentage of whole cell protein. The yield corresponds to the amount of pure protein isolated from 5×10^8 cells. n.d., not determined.

Protein	Expression levels (%)	Binding to phosphotyrosine (%)	Yield
Csk	20	80	8 mg
$Fyn^{G2A,K299M}$	2	90	1 mg
Fyn	0.1	n.d.	5 μg
p85	10	100	5 mg

200–300 mM salt. Further purification and concentration was achieved by ion-exchange chromatography on a MonoS resin (Figure 5b). By this procedure, ~ 8 mg of > 95% pure protein could be obtained from 5×10^8 cells. To apply this technique to a third, unrelated protein, we tested whether we could purify the regulatory subunit of phosphatidylinositol 3-kinase, p85 (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991), in the same manner. p85, which contains 2 SH2 domains, bound well to the phosphotyrosine resin and was eluted late in the gradient, at 900–1000 mM NaCl (Figure 6). Due to this tight association with phosphotyrosine, it was obtained as > 95% pure protein after this single step. The efficiency of this purification method for Csk, Fyn and p85 is summarized in Table 1. Thus, this method is indeed versatile, as it was applicable to three different SH2 domain-containing proteins, and should prove useful for purification of other proteins possessing this domain.

DISCUSSION

For thorough biochemical and biophysical analysis of a protein it is necessary to have large amounts of pure, soluble material. In previous purifications of Src family tyrosine kinases, proteolytic degradation has been a major problem (Varshney et al., 1986; Presek et al., 1988; Radziejewski et al., 1989; Feder and Bishop, 1990). In several reports, this problem has been successfully dealt with: purifications of high yields of full-length active protein have been obtained by conventional purification from insect cells (Lydon et al., 1992), by antibody-affinity purification from overexpressing insect cells (Morgan et al., 1991) and from human platelets (Feder and Bishop, 1990) and by affinity purification on phosphotyrosine columns as described here. In our preparations, we estimate that contamination of the pure protein by degradation products was no more than 5%. Most likely, the swiftness of the method accounts for this low amount of degradation. Furthermore, we found that the 50–55 kDa species of Fyn (representing a breakdown product) eluted earlier than full-length protein from a MonoQ column, which allowed us to enrich for the full-length protein.

When mutant versions of cSrc have been expressed in insect cells, it has been observed that mutants lacking kinase activity accumulate to higher levels than wild-type protein (Morgan et al., 1991). Mutations interfering with myristylation have been reported to reduce problems of protein degradation and also increase the yields upon purification from insect cells (Morgan et al., 1991; Lydon et al., 1992). In keeping with this, we have noted that a myristylation-deficient, kinase-inactive version of Fyn accumulates to much higher levels than does wild-type Fyn.

The method we have introduced relies on the interaction of the SH2 domain of the protein with phosphotyrosine. As the availability of the SH2 domain of Src family tyrosine kinases depends on the regulatory state of the protein, with the repressed forms not binding to low-affinity tyrosine-phosphorylated peptides, purification over a phosphotyrosine resin will yield solely the activated form of the enzyme. This may be of importance for protein crystallization studies, where a homogeneous population of proteins is expected to crystallize more readily than a mixture of different isoforms. In this respect, the use of a myristylation-deficient variant of Fyn may also be an advantage, since it is not clear whether fatty acylation occurs to full stoichiometry in insect cells. Different mutant versions of cSrc have previously been purified by antibody-affinity purification (Morgan et al., 1991). This method requires the use of high pH buffers in the elution of the enzyme. Even though the purified material displayed the same specific activity as the starting material, it is not possible to exclude that such a treatment alters the structure of non-catalytic domains of the enzyme, and in this way introduces some heterogeneity. It is worth mentioning that the elution method after affinity purification over phosphotyrosine is gentle and does not include treatment with extreme buffers, thus avoiding such uncertainties. Tyrosine-phosphorylated peptides have been previously used in the purification of phosphatidylinositol 3-kinase (Fry et al., 1992). In this case, elution of the bound enzyme was only possible under denaturing conditions. Similarly, affinity purification methods that involve tagging of proteins with epitopes for monoclonal antibodies or polyhistidines frequently employ harsh elution steps. Furthermore, the addition of amino acid sequences to a protein may be undesirable, since, for example, in the case of the Src family of tyrosine kinases it is difficult to predict what the consequences of such a modification would be on the structure or activity of the enzyme.

The method we have described here relies on the affinity of the SH2 domain for isolated phosphotyrosine. It therefore should be applicable to any SH2 domain-containing protein provided that the SH2 domain is not blocked by binding to cellular targets or involved in intramolecular interactions. We have tested this prediction for three different candidates, the Src family tyrosine kinase Fyn, its regulator kinase Csk, and the regulatory subunit of PI-3 kinase, p85. In each case, passage over the phosphotyrosine resin resulted in a highly purified form of the protein.

The elution profile of the three proteins differed markedly: whereas p85 was eluted between 750 and 950 mM NaCl as essentially pure protein, Csk was eluted at 200–250 mM NaCl in a comparatively less pure form. Most likely, this reflects differences in the affinity of the proteins for phosphotyrosine. The fact that p85 has two SH2 domains may account for the higher affinity of the protein for phosphotyrosine. As the actual affinity of the SH2 domain for a natural binding site on a tyrosine-phosphorylated protein is determined to a great extent by interactions of amino acids surrounding the tyrosine with the SH2 domain, this difference may be of little importance in a living cell. However, when isolated proteins are used to test their affinity for candidate targets *in vitro*, the fact that many SH2 domains will bind readily even to phosphotyrosine without the context of the primary amino acid sequence, as shown here for p85 and Fyn, provides a caveat for interpreting the relevance of the observed interactions.

The selectivity of the purification scheme we describe limits its application to sources where the SH2 domain of a given protein is not blocked by either intra- or intermolecular associations. Furthermore, as the number of proteins harbouring an SH2 domain in normal cells is large, a good enrichment could only be expected if sources overproducing the protein of interest were used. We noted that few other proteins bound to phosphotyrosine in insect cells. Possibly, the shut down of host-cell protein synthesis after viral infection limited the number of possible phosphotyrosine-binding molecules. Alternatively, the SH2 domain of these molecules may be engaged in associations with other tyrosine-phosphorylated insect cell proteins and therefore may not be free to bind to the resin.

Csk is a protein tyrosine kinase with remarkable specificity for members of the Src family (Okada and Nakagawa, 1988b; Bergman et al., 1992). When expressed in yeast, which have a very low natural content of phosphotyrosine, Csk cannot phosphorylate yeast proteins efficiently, whereas cSrc phosphorylates a great number of proteins (Superti-Furga et al., 1993). Indeed, so far no natural substrates other than Src family tyrosine kinases have been reported for Csk, with the possible exception of CD45 (Autero et al., 1994). Furthermore, Csk is unable to phosphorylate denatured forms of Fyn or Src, and cannot phosphorylate a peptide modelled on the C-terminal sequence of cSrc (Okada and Nakagawa, 1988a) (M. Koegl and S. A. Courtneidge, unpublished work). Apparently, the recognition of these kinases by Csk depends on characteristics of the secondary structure of the kinases. Since we found that Csk binds to a phosphopeptide modelled on the autophosphorylation site of Src family tyrosine kinases, it is tempting to speculate that this interaction serves to direct the catalytic domain of Csk towards its substrate site. However, kinase-inactive Fyn and cSrc (which are not autophosphorylated) are both good substrates for Csk, suggesting that other structural features must also be involved in determining the substrate specificity of Csk. Also, it has been reported that no complex formation between Src and Csk can be observed either *in vitro* or *in vivo* (Sabe et al., 1992). In agreement with this, we failed to detect a complex between the pure proteins *in vitro* (M. Koegl and S. A. Courtneidge, unpublished work). Thus, the complex between the two molecules, if any, has to be of a very transient nature.

Having the pure form of Fyn and Csk in hand allowed us to produce Fyn in its activated or repressed state *in vitro*, and to extend the observation that cSrc can bind to tyrosine-phosphorylated peptides only in an activated state, to the related kinase Fyn. The observation that Fyn in its repressed state did not bind to a phosphopeptide modelled on the binding site for Src family tyrosine kinases on the PDGF receptor is intriguing, since other studies have shown that the PDGF receptor phosphopeptide has a higher affinity for the Fyn SH2 domain than the phosphopeptide modelled on the C-terminal sequence of Fyn (G. Alonso and S. A. Courtneidge, unpublished work). The apparent higher affinity of the tail in the assays shown here may be an effect of its position within the three-dimensional structure of the Fyn protein, which is not displayed by isolated phosphopeptides.

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