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Expression and Purification of Src-family Kinases for Solution NMR Studies

Andrea Piserchio¹, David Cowburn², and Ranajeet Ghose^{1,3}

¹The Department of Chemistry, The City College of New York, New York 10031

²Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY 10461

³Graduate Center of the City University of New York, New York, New York 10016

Summary

NMR analyses of the structure, dynamics and interactions of the Src family kinases (SFKs) have been hindered by the limited ability to obtain sufficient amounts of properly folded, soluble protein from bacterial expression systems, to allow these studies to be performed in an economically viable manner. In this chapter we detail our attempts to overcome these difficulties using the catalytic domain (SrcCD) of c-Src, the prototypical SFK, as an illustrative example. We describe in detail two general methods to express and purify SrcCD from *E. coli* expression systems in both fully active wild-type and kinase-deficient mutant forms, allowing the efficient and cost-effective labeling by NMR-active isotopes for solution NMR studies.

Keywords

Protein tyrosine kinases; Src-family kinases; *E. coli* expression systems

1. Introduction

1. 1. Eukaryotic protein kinases as NMR targets: an overview

Protein kinases in higher eukaryotes transfer the γ -phosphate of ATP to the hydroxyl groups of specific serine, threonine (serine/threonine kinases) or tyrosine (tyrosine kinases) residues in substrate proteins. This covalent modification of the Ser/Thr/Tyr–OH moiety is a fundamental mechanism of cellular regulation and intracellular signal transduction (1–3) and has a significant role in almost every aspect of cell growth, differentiation, maturation, motility and regulated cell death. *In vivo*, the phosphorylation levels of kinase targets are tightly controlled by the opposing actions of kinases and their corresponding phosphatases (4, 5). A loss of this control due to mutations, changes in expression levels, alteration of

Address correspondence to: Ranajeet Ghose, Department of Chemistry, The City College of New York, 160 Convent Avenue, New York, NY 10031. Phone: (212) 650-6049; Fax: (212) 650-6107; rghose@sci.cuny.cuny.edu.

²¹In contrast to what was observed for the pREP4–GroESL vectors, co-transformation of both the H-MBP-3C Lyp vector and the pACYCDuet hosting SrcCD and Cdc37 does not present any difficulties, and can be performed following standard protocols with chemically competent BL21 (DE3) cells.

²⁰The k_{cat} for the phosphorylation of a substrate peptide poly-E₄Y by SrcCD was measured using a photometric assay based on the recycling of ADP and the concomitant consumption of NADH catalyzed by L-lactate-dehydrogenase (LDH) and pyruvate kinase (PK). PK regenerates ATP from ADP (a by-product of the SrcCD phosphorylation process) by converting phosphoenolpyruvate into pyruvate. LDH converts then the pyruvate into lactate by oxidizing NADH. Velocities are obtained following the time-dependent decrease of the NADH fluorescence. This method yielded a k_{cat} of $240 \pm 10 \text{ min}^{-1}$, a value comparable to that obtained previously (41).

protein-protein interactions including localization, and/or catalytic activity has been implicated in a variety of diseases including a multitude of human cancers (6–9) making protein kinases significant and established targets for anti-cancer therapies (10–13).

Eukaryotic protein, and indeed non-protein, kinases contain a highly conserved catalytic domain (CD) (14, 15) that forms a dual-lobed structure (Fig. 1) with a smaller, mainly β -sheet, N-terminal lobe (N-lobe), and a larger, mainly α -helical, C-terminal lobe (C-lobe). The catalytic activity, while wholly contained within the CD, is often regulated by additional modular domains (16) or insertions within the CD itself (17). While crystallographic analyses of both serine/threonine (18) and tyrosine kinases (19) have provided valuable insights into the regulation of their catalytic activity, several questions remain. The current hypothesis is that most protein kinases function as simple two-state switches transitioning between well-defined “active” and “inactive” conformations based on the phosphorylation state of a small number of regulatory residues. Mounting evidence suggests that this may be an oversimplification and the boundary between fully-active and fully-inactive states may be not that well-defined. Further, the simple phosphorylation-dependent switch scenario is complicated by mounting experimental evidence that hints towards the role played by long-range allosteric dynamic pathways in the regulation of kinase activity, where active-like conformations may be attained through remote interactions (20). Structural details on the nature of recognition of downstream substrates and of regulating kinases and phosphatases are still poorly defined for many of the sub-families of the human kinome. This is not unexpected, given that some of these interactions may be weak or transient, and the resultant complexes may not be amenable to crystallographic analyses.

Given this background, it would seem that solution NMR spectroscopy is tailor-made to resolve these unanswered issues about structure, dynamics and interactions involving protein kinases. Targeting eukaryotic protein kinases by using solution NMR methodologies is especially important also because of considerable interest in the pharmaceutical industry in designing small molecule inhibitors of enzymatic activity (21). In particular, the rational design of inhibitors targeting remote sites exerting allosteric control over catalytic activity in protein kinases (type III inhibitors) holds promise as a strategy for inhibiting kinase signaling in a highly selective fashion. These allosteric sites, which are more likely to be unique to specific protein kinases or kinase sub-families, could be identified by NMR studies performed on a representative sub-group of the human kinome. These studies are expected to assume greater importance as the role of yet uncharacterized protein kinases in cancer becomes clearer by using a combination of RNAi technologies in tumor cell lines and genetic knockdown studies (22–25).

Unfortunately, there have been only a handful of NMR studies reported on protein kinases. These include the receptor tyrosine kinase Eph (26), c-Abl (27), c-Src (28), protein kinase A (PKA) (29–31), and Csk (32). A large number of these targets, including PKA and Eph, for which detailed NMR analyses are available, may be expressed and purified with relative ease and are, to a large extent, well behaved in solution, in contrast to a large majority of the human kinome. NMR studies on a broader class of protein kinases have been hindered by difficulties in bacterial expression and purification of sufficient quantities of soluble, properly folded protein for economically viable labeling with NMR-active isotopes. Indeed, the only comprehensive NMR study on a non-receptor tyrosine kinase examined c-Abl expressed using the baculovirus Sf9 insect cell expression system (27). This system allows limited labeling options and is generally not an economically viable option in a non-commercial setting. Thus, to make these studies viable for a broader set of representative members of the kinome, the optimization of protocols for protein production in bacterial expression systems is required.

The problems encountered in NMR studies of eukaryotic protein kinases are exemplified by the Src-family of non-receptor tyrosine kinases (SFKs) (Fig. 1). c-Src is the prototypical representative of this nine member family (c-Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes and Yrk) (16, 33, 34). SFKs have been found to be involved in a multitude of human cancers including those of the breast, colon, gastrointestinal tract, lung and in leukemias, lymphomas and myelomas (35). In particular, the catalytic activity of c-Src is highly elevated in a majority of human colon cancers (36). SFKs in general, and c-Src in particular, are therefore key targets for the design of drugs to treat a wide range of human cancers (37). The catalytic activity of c-Src, and indeed of other SFKs, depends on the phosphorylation state of two conserved tyrosine residues (chicken c-Src numbering used throughout): Tyr416 (activation) and Tyr527 (suppression). Phosphorylation occurs at Tyr416 through an intermolecular mechanism, while Tyr527 is phosphorylated by another family of non-receptor tyrosine kinases that is comprised of two members: C-terminal Src kinase (Csk) and Csk-homologous kinase (Chk) (38, 39).

Several researchers have utilized a variety of systems, including insect cells (40), yeast (*S. pombe*) (41) and platelets (42) to express c-Src. Weijland *et al.* (41) used a *S. pombe* based strategy to express the catalytic domain of c-Src by co-expressing the catalytic domain of the tyrosine phosphatase PTP-PEST to reduce catalytic activity and the resultant cytotoxicity. As an extension of this strategy, Seeliger *et al.* (43) have reported the *E. coli* expression of the catalytic domains of c-Src and c-Abl by co-expressing the downregulating YopH phosphatase (43). However, multiple issues, including poor sample homogeneity, multiple phosphorylation states (44), sample aggregation or degradation are often encountered when using these strategies. *E. coli*, the system of choice for production of material amenable to analysis by solution NMR spectroscopy, is particularly affected by these drawbacks (28). The expression and purification of the catalytic domain of c-Src (SrcCD) from *E. coli* for NMR studies was hindered by several major complications that include:

1. Cytotoxicity resulting in poor cell growth after induction.
2. Protein misfolding leading to the accumulation of large amounts of insoluble material in inclusion bodies resulting in vanishing low yields of properly folded soluble protein. Attempts to refold these misfolded aggregates using chaperones *in vitro* have also not yielded promising results (Piserchio *et al.* unpublished results).
3. Heterogeneously phosphorylated protein, most notably on the activating tyrosine residue (Tyr416), the auto-phosphorylation of which constitutes the central step in kinase activation.

Resolution of these issues is absolutely necessary for NMR, with its ability to provide both structural and dynamic information over a wide range of timescales, to play a role in studies of these key signaling molecules for drug discovery (45). Here we describe our efforts towards alleviating these issues by optimal choice of constructs, the use of solubility-enhancing tags, co-expression with chaperones and carefully optimizing overexpression conditions (28). These methods should provide a useful guide for the bacterial expression and purification of similar protein kinases for NMR studies.

1. 2. Expression of SrcCD in *E. coli* as an MBP fusion protein: the overall expression strategy

The first strategy that we utilized for the bacterial expression of c-Src involved (1) the addition of an N-terminal MBP fusion tag to enhance both solubility and folding; (2) the co-expression of GroEL and GroES chaperones to further facilitate the accumulation of properly folded protein, an idea originally proposed by Cole (46); and (3) a decrease in the

growth temperature after induction to 15°C thus reducing the rapid accumulation of large quantities of proteins and macromolecular crowding effects that tend to facilitate aggregation.

MBP is widely used to enhance the production of fused proteins through a mechanism that is far from clear. Several possible mechanisms have been proposed including the formation of large vesicular aggregates with the misfolded or unfolded proteins contained inside (47) or a general chaperoning effect (48). Our initial attempts to produce the catalytic domain of the so-called “kinase-dead mutant” bearing a Lys→Met mutation (SrcCD_{K295M}) (49) in M9 minimal medium showed that in fact, just the introduction of a N-terminal MBP fusion, while not eliminating the presence of protein in the insoluble inclusion bodies, drastically increased the amount of soluble material. However, the majority of the protein produced in this fashion found in solution consisted of soluble aggregates, presumably formed by misfolded material. Observation of similar phenomena has added credence to the speculation that MBP fusions can form micellar-like systems, formed by unfolded material in the core, and by the soluble MBP domain on the surface (47). Though the relatively small amount of properly folded SrcCD_{K295M} that was obtained by these preparations allowed us to collect the first ¹⁵N-HSQC spectra of SrcCD_{K295M}, the quantity of soluble, properly folded SrcCD obtained using this procedure was not sufficient for detailed NMR analysis. This was because a large number of experiments, collected over long periods of time, are required for the resonance assignment process, necessitating multiple NMR samples produced using expensive labeling supplies. Nevertheless, the observation that material suitable for NMR experiments could be obtained from an *E. coli* expression system was indeed encouraging.

The largest improvement in the yield of properly folded SrcCD_{K295M} came when the N-terminal MBP fusion system was transformed into *E. coli* cells that overexpress GroEL and GroES chaperones. Cole and coworkers have shown that overexpression of these bacterial chaperones allow the purification of small amounts of SrcCD_{K295M} from *E. coli* (49). We found that a more substantial improvement, also achievable in the minimal media used for NMR sample preparation, can be obtained by integrating the MBP fusion and the GroELS systems. Furthermore, the same approach could also be used to grow wild-type SrcCD (as opposed to SrcCD_{K295M}), albeit with lower yields, with minimal alteration of the overall protocol. The introduction of the chaperones, however, did not completely eliminate the formation of soluble aggregates mentioned above. However, we noticed that, while these undesired aggregates bound to the amylose columns that are routinely employed to purify MBP fusion proteins, they were unable to bind Co⁺² affinity columns despite the presence of an N-terminal His₆-tag, most likely because the His₆-tag was occluded in the misfolded species. This fortuitous scenario allowed us to use metal affinity chromatography to efficiently separate the soluble aggregates from the properly folded SrcCD.

When a target protein is expressed as an MBP fusion, a critical step prior to NMR analyses is the removal of the bulky MBP tag from the fusion. SrcCD can be efficiently separated from the MBP tag by inserting a thrombin cleavage site between the two components of the fusion construct, although some care has to be taken to avoid protein degradation in the presence of thrombin. We found that resins that bind the N-terminal end of the cleaved fusion (metal-chelating for His₆ or amylose for the MBP tag) cannot be efficiently used to isolate SrcCD, given the tendency of the kinase to bind nonspecifically to these columns. We then found that separation of MBP and SrcCD can be carried out using ion exchange chromatography (Fig. 2). The catalytically compromised SrcCD_{K295M} could be efficiently separated from cleaved MBP in this fashion. Interestingly, during purification we found that two forms of SrcCD_{K295M} (one selectively phosphorylated at the activating Tyr416 and the other not; confirmed by Western blot against anti-pTyr antibodies and MS/MS analysis) (28)

were obtained as two distinct peaks in the elution chromatogram (Fig. 2). This last observation was surprising to some extent, because Q columns do not usually have sufficient resolution to separate identical proteins with unit changes in charge states. Crystallographic and computational analyses *e.g.* (50, 51) have revealed that activating phosphorylation alters the overall conformation of the catalytic domain, thus the protein surface that interacts with the column is also likely altered. This feature, we believe, allows the separation of the phosphorylated and non-phosphorylated species using ion-exchange chromatography. We provide below a step-by-step protocol for the purification of SrcCD as an MBP fusion in **Section 3. 1.**

1. 3. Expression of SrcCD in *E. coli.* using the kinase-specific chaperone, Cdc37: the overall general strategy

The MBP/GroELS based protocol, though complex in the nature of its purification scheme, results in high yields of SrcCD purified to homogeneity (up to 700 nmoles per liter of culture). However, the biggest disadvantage lies in the fact that the catalytic domain of c-Src can only be purified either as a kinase-deficient mutant (SrcCD_{K295M}) or in wild-type form in complex with a high-affinity inhibitor (*see Section 3. 1. 2*), using this strategy. This prevents access to the full range of catalytically relevant conformations and motional modes, in spite of having the ability to purify both constructs (mutant and wild-type) with the activating Tyr416 in the unphosphorylated and selectively phosphorylated forms. Thus, we developed an alternative strategy to purify wild-type SrcCD that displays full catalytic activity in solution.

Our experiences seem to indicate that proper folding appears to be the main hindrance to SrcCD production in bacterial expression systems. In eukaryotes, the folding of several protein kinases including c-Src, occurs with the assistance of the Hsp90 chaperone machinery (52–54). This process is further assisted by an essential kinase-specific co-chaperone Cdc37/p50, which can independently bind both the kinase target and the Hsp90 chaperone, thus approximating the kinase and the chaperone machinery, and stabilizing the transient ternary complex (55, 56). Indeed, ternary complexes of Hsp90, v-Src (a transforming viral form of c-Src that contains a tail truncation and lacks Tyr527) and Cdc37 (57) have been identified.

We found that co-expressing the Cdc37 co-chaperone (the plasmid encoding Cdc37 was a kind gift from Avrom Caplan, CCNY) with wild type SrcCD leads to a properly folded soluble kinase domain even in the absence of the N-terminal MBP fusion protein (Fig. 3). Consequently the His₆-tagged kinase can be purified easily using metal affinity chromatography followed by size exclusion chromatography. Thrombin cleavage and ion-exchange chromatography, steps that can both lead to substantial losses in material, become unnecessary. Although the yields are somewhat lower, the methodology is less time consuming, and the wild-type kinase domain can be isolated without the use of an inhibitor, allowing access to the full complement of structural and dynamics changes that accompany activation. The detailed protocols used are described at length in section 3. 2.

2. Materials

Most of the materials utilized in the experiments described below are routinely used in NMR laboratories for protein expression and purification.

2.1. Expression vectors and competent cells

1. H-MBP-3C vector (58): A kind gift from Dr. Kaushik Dutta, New York Structural Biology Center. (*see Note 1*)

2. pACYCDuet vector: for simultaneous expression of two proteins (*see* Note 2): SrcCD and Cdc37 should be cloned into this vector following the guidelines described below.
3. 0.1 cm gap sterile electroporation cuvette for bacteria.
4. Electroporator (*see* Note 3).
5. pREP4-groELS electrocompetent BL21 (DE3) cells. (*see* Note 4).
6. BL21 (DE3) T1 competent cells.

2.2. Media for bacterial growth

1. LB (Luria Bertani) Miller medium: Dissolve 20 g of LB powder in 1 L of water, and sterilized by autoclaving. Store at room temperature.
2. M9 salts: 47.9 mM anhydrous Na₂HPO₄ (6.8 g/L), 22.0 mM anhydrous KH₂PO₄ (3 g/L) and 8.6 mM NaCl (0.5 g/L), sterilized by autoclaving. Store at room temperature
3. Ampicillin stock (1000X): 100 mg/mL Ampicillin in water. Store at 4 °C.
4. Kanamycin stock (1000X): 50 mg/mL Kanamycin in water. Store at 4 °C.
5. Chloramphenicol stock (1000X): 35 mg/mL Chloramphenicol in Isopropanol. Store at 4 °C.
6. LB plates: Mix 40 g/L of LB Miller agar and autoclave the suspension, let the material cool to ~ 55 °C and add the desired antibiotic (100 mg/L Ampicillin, or 50 mg/L Kanamycin, or 35 mg/L Chloramphenicol) or combination of antibiotics (100 mg/L Ampicillin and 50 mg/L Kanamycin, or 100 mg/L Ampicillin and 35mg/L Chloramphenicol)
7. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG): Prepare in water and store at 4 °C.
8. Metal Solution I (1000X): 1 mM MnCl₂·4H₂O, 0.2 mM CoCl₂·6H₂O, 2 mM ZnCl₂, 1 mM CuSO₄·5H₂O, 50 mM H₃BO₄, 0.001 mM Na₂MoO₄. The compounds are added in the order listed above, dissolving each compound completely before adding the following one.
9. Metal Solution II (1000X): 1 mM FeSO₄, filter sterilize and store at 4 °C.
10. MgSO₄ stock (500X): 1 M MgSO₄, autoclave and store at room temperature.
11. CaCl₂ stock (1000X): 0.1 M CaCl₂, autoclave and store at room temperature.
12. Vitamin solution (100X): Prepare 10 mL aliquots in 15 mL centrifuge tubes, and store at −20 °C (*see* Note 5).

¹The H-MBP-3C vector contains a thrombin cleavage site and features a maltose binding protein (MBP) tag to enhance solubility and a His₆ sequence to facilitate protein purification using a metal affinity chromatography. The protease 3C site described in the original publication (58) has been replaced by a thrombin site in the present case.

²The pACYCDuet vector was purchased from Novagen (EMD Biosciences).

³We used an Eppendorf 2510 electroporator.

⁴We observed that the transformation of the pREP4-groESL vector was often inefficient, and therefore we avoided the simultaneous double transformation of pREP4-groESL and H-MBP-3C vector encoding SrcCD, by preparing a stock of electrocompetent cells pre-transformed with the former. Electrocompetent cells were preferred over chemically competent cells, due to their ease in preparation and high efficiency of transformation. These cells were prepared by streaking out commercial BL21 (DE3) competent cells on an LB plate and incubating the plate overnight at 37°C. From this, electrocompetent cells were prepared using standard protocols.

⁵We used Kao and Michaylchuk vitamin solution (100X) purchased from Sigma.

13. All material required to label the overexpressed proteins with NMR-active isotopes, namely $^{15}\text{NH}_4\text{Cl}$, $^{13}\text{C}_6$ -Glucose, $^2\text{H}_7$ -Glucose, $^2\text{H}_7$, $^{13}\text{C}_6$ -Glucose, CELTONE[®] powders ($[\text{U-}^{15}\text{N}, ^2\text{H}]$ and $[\text{U-}^{15}\text{N}, ^2\text{H}, ^{13}\text{C}]$, see Note 6), ^{14}N , ^1H , ^{12}C , ^{13}CO amino-acids. The material are weighed and carefully dissolved in the minimum amount of preparation medium (40 mL for 2.5 g of glucose or 0.5 g of NH_4Cl), sterile filtered and added immediately to the medium. Powders are stored at room temperature.
14. Anhydrous dextrose.

2. 3. Protein purification

1. Human α -thrombin, store at -80°C .
2. Empty 20 mL bed volume chromatography columns.
3. Pre-equilibrated cobalt resin: Resuspend the cobalt-based beads (*see* Note 7) for metal-affinity purification of His₆-tagged proteins by gently inverting the bottle. Typically 4 mL of bed volume is used for every 0.5 L growth (2 mL of slurry provides 1 mL of bed volume). Once resuspended, pipette the slurry into a 20 mL empty column until all the methanol is washed away and the resin settles. Gently wash the column with three bed volumes of deionized H_2O followed by three bed volumes of lysis buffer. Resuspend the resin in 1–2 mL of lysis buffer (by gently inverting the closed column) and add to the cell lysate.
4. Lysis buffer: 20 mM Tris, pH 7.8 (titrated with 36.5–38 % HCl), 0.5 M NaCl, 1 % Triton X-100, 0.1 % β -mercaptoethanol. The buffer is prepared without β -mercaptoethanol and Triton-X, and stored at room temperature with these added immediately before usage.
5. Q columns (5 mL): for ion-exchange chromatography (*see* Note 8).
6. Ion-exchange chromatography buffer A: 20 mM Tris, pH 7.4 (titrated with 36.5–38 % HCl), 10 % glycerol. Store at room temperature.
7. Ion-exchange chromatography buffer B: 20 mM Tris, pH 7.4 (titrated with 36.5–38 % HCl), 1 M NaCl, 10 % glycerol. Store at room temperature.
8. Dialysis buffer: (before size-exclusion chromatography): 20 mM Tris, pH 7.5 (titrated with 36.5–38 % HCl), 150 mM NaCl, 1 mM EDTA and 1 mM DTT (Dithiothreitol), prepared immediately before use.
9. Size exclusion chromatography buffer: 50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 1 mM DTT. 4.82 g of $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ and 2.14 g of Na_2HPO_4 are added to 1 L of water, the pH is adjusted if necessary using 36.5–38 % HCl or 1 M NaOH, The DTT is added immediately before use and the rest stored at room temperature.
10. Gel filtration column (*see* Note 9): Pre-equilibrate the column with 1.5 volumes of size exclusion chromatography buffer. The typical flow rate is 0.5 mL min^{-1} .

⁶CELTONE[®] is a rich growth medium for bacteria derived from algal extracts. It may be purchased from Cambridge Isotopes Laboratories.

⁷We used TALON beads purchased from Clontech.

⁸We used HiTrap Q columns from GE Healthcare Biosciences Corp.

⁹We used a HiPrep 16/60 Sephacryl S-100 column from GE Healthcare Biosciences Corp. All protein purification was carried out using an Akta Explorer 100 from GE Healthcare Biosciences Corp.

11. AP23464: 10 mM in DMSO. The high-affinity kinase inhibitor (59) is a kind gift from Dr. David Dalgarno, Ariad Pharmaceuticals. Dissolve 4.7 mg of compound in 1 ml dimethyl sulfoxide.
12. 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF)
13. Imidazole: 20 mM Tris, 0.5 M NaCl, 1 M Imidazole. Dissolve 1.2 g Tris, 14.61 g NaCl and 34.04 g of Imidazole in ~ 450 ml H₂O. Adjust the pH at 7.8 using 36.5–38 % HCl, bring the solution to 0.5 L volume using a graduated cylinder, and adjust again the pH to 7.8 using 36.5–38 % HCl or 1 M NaOH. Cover the bottle with aluminum foil and store at room temperature.
14. Cleavage buffer: 20 mM Tris, pH 7.8 (titrated with 36.5–38 % HCl), 150 M NaCl, 4 mM β-mercapto-ethanol. Add β-mercapto-ethanol before cleavage.
15. 1 M Sodium Hydroxide: dissolve 40 g of NaOH in ~ 950 ml of water, bring to 1 L volume using a graduated cylinder.
16. EDTA: 20 mM Tris, 0.5 M EDTA. Dissolve 1.2 g of Tris and 73 g of Ethylenediaminetetraacetic acid in 450 ml of water, adjust the pH to 8.0 using 1 M NaOH, bring to volume with a graduate cylinder and adjust the pH to 8.0 using 36.5–38 % HCl or 1 M NaOH.
17. AEBSF: 41.7 mM solution, 1 mL aliquots. Dissolve 10 mg 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride in 1 mL water, store at –20 °C.

3. Methods

3.1. E. coli expression and purification of the catalytic domain of c-Src as an N-terminal MBP fusion

The detailed protocol provided below is for the bacterial expression and purification of the catalytic domain of the kinase deficient construct (SrcCD_{K295M}) or the wild-type domain in the presence of a high-affinity inhibitor.

3.1.1. Overexpression of SrcCD

1. Thaw a tube containing the pREP4-groELS electrocompetent BL21 (DE3) cells, on ice (*see* Note 4).
2. Add ~20 ng of H-MBP-3C vector, encoding SrcCD, to the electrocompetent cells. Transfer the contents into a pre-chilled electroporation cuvette and incubate for 2 minutes on ice.
3. Transform the cells with a 6 ms pulse at 1730 V, and add 960 μL of SOC or LB medium, pre-warmed to 37 °C. Transfer into a sterile 15 mL conical centrifuge tube and shake at 250 rpm for 1 hour in an incubator set to 37 °C.
4. Plate ~100 μL of transformed cells on a pre-warmed (37 °C) LB plate containing both Kanamycin and Ampicillin resistance (50 mg/L and 100 mg/L, respectively). Incubate overnight at 37 °C.
5. Transfer the plate to 4 °C for storage.
6. Autoclave 0.5 L of M9 salts in a 2 L flask, transfer 20 mL of this solution into a 50 mL conical tube. From the stock solutions, add 20 μL of CaCl₂ (final concentration: 0.1 mM), 40 μL of MgSO₄ (final concentration: 2.0 mM), 20 μL of Metal Solutions I and II, and 200 μL of Vitamins solution (1%), 20 μL of Ampicillin and Kanamycin (final concentration: 100 and 50 mg/L, respectively). Finally, add 0.1 g (5 g/L) of anhydrous dextrose and 20 mg (1 g/L) of ammonium

chloride. Dissolve by agitation or vortexing, and filter into a pre-autoclaved 125 mL flask using a sterile 50 mL syringe and a sterile 0.22 μm , 33 mm filter.

7. Scoop a few colonies from the transformed plate using a sterile loop, transfer into the M9 medium and incubate overnight in a shaker (250 rpm) at 37° C.
8. Using a sterile culture pipette, transfer ~20 mL of the M9 salt solution into a sterile 50 mL centrifuge tube. Use this solution to dissolve 2.5 g of dextrose and 0.5 g of ammonium chloride (final concentration: 5 g/L and 1 g/L, respectively). Then add 5 mL of vitamin solution, dissolve by shaking or vortexing, and filter the entire content into the original 2 L flask. Add 500 μL of CaCl_2 and MgCl_2 stock solutions (final concentration: 0.1 mM and 2.0 mM, respectively), 500 μL of Metal Solutions I and II, 500 μL of Ampicillin and Kanamycin (final concentration: 100 and 50 mg/L, respectively), to the flask.
9. Add the 20 mL overnight culture directly into the 0.5 L of M9 medium. Incubate at 250 rpm at 37° C.
10. After 1 hour, check the OD_{600} every 30 minutes; when the OD_{600} reaches ~ 0.8, add 100 μL of the IPTG stock solution (final IPTG concentration: 200 μM , *see* Note 10). Keep the flask at 37 °C for about 30 minutes (*see* Note 11).
11. Transfer the flask into a second shaker with the temperature set to 15 °C, and incubate at 250 rpm overnight (*see* Note 12).
12. Centrifuge the cells at 4900 g for 20 minutes at 4 °C. Store the cell pellet at –80 °C.
13. For ^{15}N , ^{13}C enrichment, replace $^{14}\text{NH}_4\text{Cl}$ and dextrose in steps (6) and (8) described above, with $^{15}\text{NH}_4\text{Cl}$ and [$U\text{-}^{13}\text{C}$]-glucose, respectively.
14. Under normal circumstances, for uniform ^{15}N , ^{13}C , ^2H enrichment, grow the cells in M9 medium in which H_2O is replaced by D_2O . However, in the case of SrcCD, we found that the introduction of $^2\text{H}_2\text{O}$ dramatically reduces the yields. Therefore we follow the approach developed by Fiaux and co-workers (60), where a pool of ^{15}N , ^{13}C , ^2H -labeled amino acids were used to supplement M9 medium prepared in H_2O . In this case: In step (6) above, replace $^{14}\text{NH}_4\text{Cl}$ with $^{15}\text{NH}_4\text{Cl}$; in step (8), above, replace $^{14}\text{NH}_4\text{Cl}$ with $^{15}\text{NH}_4\text{Cl}$, and use 0.5 g of dextrose (1g/L instead of 5 g/L) and add 0.75–1.0 g of ^{15}N , ^{13}C , ^2H -labeled algal lysate medium (*e. g.* CELTONE® 1.5–2.0 g/L).

3.1.2. Protocol for the purification of SrcCD from the MBP fusion protein

1. Lyse cells using a French Press (1100 psi), followed by centrifugation at 16000 g for 20 minutes at 4°C.

¹⁰Lower IPTG concentrations are recommended at low temperature to avoid toxicity.

¹¹This helps to increase the final yield by boosting mRNA production, while most protein is synthesized during the following lower temperature growth. In the first hour after induction bacteria mainly activate mRNA transcription, while actual translation is still minimal.

¹²All bacterial growth post-induction (after the initial ½ hour at 37°C, *see* Note 11) was carried out at a low temperature (15°C). It is well known that low temperatures decrease the degradation rates of unstable proteins; in the case of SrcCD, we observed that low temperature growth is essential for the production of soluble, properly folded SrcCD. We speculate that SrcCD folding is a slow process that is in competition with the formation of aggregates both soluble and insoluble. In our protocol both the N-terminal MBP fusion and the GroEL/GroES chaperones (or the Cdc37 co-chaperone) provide assistance in stabilizing the nascent chain and protect it from aggregation. Lower temperatures likely assist this process by reducing the rates of protein synthesis and aggregation.

2. Check the pH of the supernatant (*see* Note 13), if it is below 7.5, adjust by adding small amounts of solid tris-hydroxymethyl-aminomethane (Tris).
3. Transfer pre-equilibrated Co-resin into the centrifuge tube containing the lysate supernatant, and incubate for about 1 hour under rotation or slight agitation at 4 °C.
4. Pour the lysate and beads into an empty column. Eluting the lysate and wash the column with four bed volumes of lysis buffer and with two bed volumes of lysis buffer containing 3 mM imidazole (1 μ L Imidazole per ml of lysis buffer).
5. Elute the protein with four bed volumes of the lysis buffer containing 300 mM imidazole (combine 3 volumes of Imidazole with seven volumes of lysis buffer).
6. Add 10 mM EDTA (*see* Note 14) to the eluate *prior* to dialysis (add 20 μ L EDTA solution per mL of eluate), then dialyze against cleavage buffer at 4 °C.
7. Determine the concentration of the fusion protein by measuring the OD₂₈₀ using 119,100 M⁻¹ cm⁻¹ as the extinction coefficient.
8. Add 5 units of α -thrombin per mg of fusion protein to the solution, immediately add β -mercaptoethanol to a final concentration of 4 mM. Cleave overnight at 4 °C.
9. Check for completion of the cleavage reaction by running an SDS-PAGE gel. Add more thrombin if necessary. When the cleavage is complete, add solid DTT and the protease inhibitor, AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride) to final concentrations of 5 mM and 100 μ M, respectively. Add AP23464, a high-affinity kinase inhibitor, to the cleavage buffer to a final concentration of 100 μ M for wild type SrcCD.
10. Dilute the completed reaction 5-fold into ion exchange chromatography buffer A, and load the resulting sample onto a Q column (ion-exchange) at a flow-rate of 4 mL/min (the volume at this stage is likely to be large, possibly more than 100 mL; *see* Note 15).
11. Elute the cleaved kinase by running a salt gradient from 0 to 300 mM NaCl in 120 minutes using a flow-rate of 1 mL/minute. Unphosphorylated SrcCD_{K295M} appears at around 15.4 mS/cm and SrcCD_{K295M} phosphorylated at Tyr₄₁₆ appears at around 16.9 mS/cm. Wild type SrcCD bound with the kinase inhibitor, AP23464, appears at around 20.4 mS/cm. MBP elutes at around 8–10 mS/cm, multiple peaks may appear due to non-specific phosphorylation (*see* Note 16).

3.2. Expression and purification of the fully active, wild- type catalytic domain of c-Src in *E. coli*

We provide below a step-by-step protocol for the expression and purification of wild-type SrcCD without the use of an N-terminal MBP tag. The SrcCD obtained using this protocol is

¹³We noticed that after long induction times at low temperature, *E. coli* cells release significant amounts of acid molecules into the lysis buffer. This is, in some instances, sufficient to overcome the buffering power of the solution leading to a pH below 7.0, compromising efficient binding of the His₆ tag to the metal affinity column.

¹⁴EDTA is introduced to chelate Co²⁺ cations. Metal leaks from the resin can be caused by mechanical stress and by high Imidazole concentrations. If not chelated by EDTA, Co²⁺ can be difficult to eliminate by dialysis, as it can bind the His₆-tag as well as well as the metal binding pockets in SrcCD if left in the sample. Co²⁺ forms a reddish precipitate once a substantial amount of reducing agent has been added (interfering with the remaining purification process), while the Co²⁺ remaining in solution, being paramagnetic, degrades the quality of the NMR spectra.

¹⁵We normally do not load more than 15 mg of fusion protein into the ion exchange column at a given time.

¹⁶Unlike the catalytically compromised mutant, wild type SrcCD did not elute once loaded on the Q column. Presumably the wild-type protein is less stable under conditions of low ionic strength used for sample loading, and precipitates on the column. We found however, that this construct could also be successfully loaded and eluted using the protocol described above by adding a high-affinity kinase inhibitor (59) after cleaving the fusion protein.

fully active and does not require a kinase inhibitor to allow purification to homogeneity. To implement this protocol, first SrcCD and Cdc37 have to be cloned into a pACYCDuet vector, (Chloramphenicol resistant) using standard procedures. SrcCD should be inserted in the first cloning site using the *EcoRI* and *SalI* restriction enzymes (resulting in a N-terminal His₆-tag for SrcCD, see Note 17), Cdc37 in the second site using *XhoI* and *NdeI*. SrcCD expression was also tested with and without the concomitant co expression of a third protein, the tyrosine phosphatase, Lyp (61) (a kind gift from Ronald Seidel, Albert Einstein College of Medicine). Lyp selectively dephosphorylates the activating Tyr residue in SFKs (specifically Tyr394 in Lck), and was obtained in a H-MBP-3C Ampicillin resistant vector (see Note 1).

1. If using electrocompetent cells, transfer ~20 ng of pACYCDuet vector into the Eppendorf tube containing the BL21(DE3)T1 electrocompetent cells. Transfer the contents of the Eppendorf tube into a pre-chilled electroporation cuvette and incubate for 2 minutes on ice (see Note 18). If also overexpressing the phosphatase, then transfer both vectors (pACYC and H-MBP-3C, 20 ng each) into the cuvette at the same time (see Note 19)
2. Follow steps 4–14 of **Section 3.1.1** but use Chloramphenicol (35 mg/L) and Ampicillin only if overexpressing the phosphatase. This applies to step (4) (Chloramphenicol, or Ampicillin and Chloramphenicol double resistant plates) and step (6) (Chloramphenicol, or Ampicillin and Chloramphenicol added to the M9 medium)
3. Purify the protein following steps 1–6 of **Section 3.1.2**. Note that the final purified protein retains the His₆-tag. If the tag needs to be removed, a thrombin cleavage site could be inserted at step 1 in the SrcCD forward primer (described in Note 10). Then steps 7–9 from **Section 3.1.2**. would also apply. Note that, in our construct, the MBP-phosphatase fusion protein has an N-terminal His₆ tag and co-elutes with the kinase.
4. After elution, dialyze the protein(s) against dialysis buffer.
5. Concentrate the protein(s) down to 2 mL and inject into a size exclusion column. Note that the phosphatase elutes in the void (presumably due to some level of self-association) while the kinase elutes consistently at the volume expected for its molecular weight, and are both readily isolated.

Both methods of expression (with and without co-expression with Lyp) lead to the isolation of active, wild-type SrcCD (confirmed by analysis of kinetics, see Note 20). However, a higher final OD₆₀₀ and overall yield of purified protein was observed when the kinase was co-expressed with Lyp phosphatase (~190 nmoles per L of culture).

3.3. Conclusions

We have described how careful optimization of overexpression protocols allows the expression and purification of the catalytic domain of c-Src both in mutant, as well as active wild-type forms using *E. coli* expression systems. In particular we developed two related

¹⁷For SrcCD (human) we used the following primers:

¹⁸Forward: GGTGGTGAATTCGTCCAAGCCGAGACTCAGGG

¹⁹Reverse: GGTGGTGGTCGAC CTAGAGTTCTCCCCGGGCTGGTA.

For Cdc37 we used the following primers:

Forward: GGTGGTGCATATGGTGGACTACAGCGTGTGGGAC

Reverse: GGTGGTGCTCGAGTCACACTGACATCCTTCTCATCGCC.

²⁰The electrocompetent cells were home made and derived from the commercial BL21 (DE3) T1 expression system (chemical competent). We expect that transformation directly into the commercial electrocompetent cells would produce equivalent results.

strategies, the first employs both an N-terminal MBP fusion and co-expression with the GroEL and GroES chaperones; while the second, by taking advantage of the higher specificity of the Cdc37 co-chaperone for the nascent c-Src chain, does not require the production of SrcCD as an MBP fusion protein. Both methods, present advantages and disadvantages. In particular, the first method leads to higher yields, but it is more efficient in producing the catalytically compromised (K295M) mutant compared to the wild type protein. The wild-type protein can only be purified in the presence of a high-affinity kinase inhibitor when using this method.

The second method produces lower yields; however it also less labor intensive and more importantly, allows the production of the catalytic domain in active, wild-type form. Given the higher yields, the first method is probably more useful when SrcCD is required in large amounts for studies such as those to determine specific protein-protein interactions that do not require the intact catalytic properties of the kinase. However, given that the MBP-dependent protocol also produces a considerable amount of soluble misfolded aggregates that have not been observed using the Cdc37-dependent protocol, we believe that the latter may be better suited for “in-cell” NMR studies in *E. coli* (62–64). Obviously, the latter protocol is *required* when wild-type, fully active SrcCD needs to be produced.

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Figure 1.

(a) Domain arrangement in inactive full-length c-Src (PDB ID: 2SRC). The residues comprising the linker between the SH2 and the catalytic domain (CD), which engages the SH3 domain, is shown in ball-and-stick representation, as are the residues that comprise the C-terminal tail. The regulatory tyrosine residues in the activation loop (Y416, unphosphorylated in inactive c-Src) and the C-terminal tail (Y527, phosphorylated in inactive c-Src) are also shown in ball-and-stick representation. The ATP-analog, ANP (phosphoaminophosphonic acid-adenylate), bound to the ATP-binding site, is also shown in ball-and-stick representation. (b) Expanded view of SrcCD with the N-lobe and C-lobes indicated. Important regulatory elements that include the activation loop, Gly-rich loop, the HRD (that precedes the catalytic loop) and DFG motifs are also indicated. Y416 is shown in a ball-and-stick representation and the α C helix is indicated by a dashed ellipse. The ligand ANP is also shown bound to the ATP-binding pocket.

Figure 2.

Production and purification of SrcCD_{K295M}. (a) SDS-PAGE gel (20 %) showing different steps in the purification process: lane 1 – GroEL and GroES (expressed alone), lane 2 -cell lysate supernatant, lane 3 - cell lysate pellet, lane 4 - metal affinity column flow through, lane 5 - metal affinity column eluate, lane 6 – product after thrombin cleavage (MBP – higher molecular weight and SrcCD_{K295M} – lower molecular weight), lane 7 – SrcCD_{K295M} after Q-column purification. (b) Effects of controlled phosphorylation and dephosphorylation of SrcCD_{K295M} on the Q column elution profiles. Dashed line: sample pre-incubated with catalytic amounts of commercial wild-type c-Src and Mg²⁺/ATP. Dark solid line: sample pre-incubated with alkaline phosphatase (non-specific phosphatase). Light solid line: sample pre-incubated with Lyp phosphatase (specific for phosphorylated Y416). The identity of pSrcCD_{K295M} was confirmed by immunoblots using anti-pTyr antibodies and MS/MS following trypsin digestion.

Figure 3.

(a) SDS-PAGE gel (20 %) showing the various steps in the purification of wild-type SrcCD (His₆-tagged): lane 1 - cell lysate supernatant, lane 2 - cell lysate pellet, lane 3 - metal affinity column flow-through (unbound material), lane 4 - metal affinity column eluate, lane 5 - cSrc after purification using gel filtration. The positions of Cdc37, MBP-Lyp and SrcCD in lanes 3, 4 and 5, respectively, are highlighted by boxes. Samples in lanes 4 and 5 were concentrated by a factor of 10. (b) Size exclusion column profile of the eluate from the metal affinity column purification. (c) Immunoblots of phosphorylated wild-type SrcCD (preincubated with 2 mM ATP and 10 mM Mg²⁺) with anti-pTyr antibody. All immunoblots were visualized using the Bio-Rad ChemiDoc system. Lane 1 - phosphorylated SrcCD, lane 2 - phosphorylated SrcCD after incubation with alkaline phosphatase.