Experimental Methods

Methods for the recombinant expression of active tyrosine kinase domains: guidelines and pitfalls

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Insect cell expression

Cloning and virus preparation

All constructs were inserted into pFASTBAC (ThermoFisher) vector, which was modified to include an N-terminal EGFP and 3C protease recognition site. The FERM and Kinase domain of human FAK (31-687) and human Src (86-529) containing SH2, SH3 and Kinase domains without C-terminal Tyr530 were cloned using the CPEC method (Quan & Tian, 2009). After sequence verification, each construct was transformed into DH10Bac (ThermoFisher) competent cells for the creation of bacmids. White colonies indicating a disrupted *lacZ* gene were selected and incubated overnight at 37°C. Bacmids were purified with the QiaPrep Miniprep kit (Qiagen) and transfected into SF9 (Expression System) with the Cellfectin II reagent (ThermoFisher) for virus production. Virus amplification was evaluated through EGFP fluorescence in cells without titration and virus was repeatedly harvested and used for infection until the multiplicity of infection was satisfactory. Amplified virus was stored at 4°C until usage.

Protein expression and purification from insect cells.

SF9 cells were grown and maintained within ESF921 media (Expression system) at 27°C and 120 rpm. When the cell density reached 1.2 –1.5x10^6 cells/ml, cells were infected with each

virus and incubated for 72 h or until their viability fell below 70%. Cells were harvested by centrifugation (2000 x g, 20 min., 4°C) and pellets were washed three times with pre-chilled PBS buffer and re-suspended with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM TCEP and 5% glycerol) supplemented with EDTA-free inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride and Benzonase (EMD Millipore). Re-suspended cells were lysed by sonification (20%, for 2 min with 5 s *on* and 10 s *off* cycle) and the soluble fraction was separated from cell debris using high speed centrifugation (50,000 x g, 1 h, 4°C). The supernatant was collected and incubated with Sepharose 4B (GE Healthcare) resin charged with GFP-Nanobody (Kubala, Kovtun, Alexandrov, & Collins, 2010) and the resin was washed using excessive volume of lysis buffer. The target proteins were eluted by on-gel digestion with HRV 3C protease and further purified by size-exclusion chromatography on a Superdex200 10/300 GL (GE Healthcare) column.

E. coli **expression**

Construction of KD plasmids

Kinase catalytic domains were defined as in (Camacho-Soto et al., 2014): residues 211-512 of Lyn (Uniprot P07948), 234-536 of Src (Uniprot P12931) and residues 1-336 of a FAK isoform (genbank AAH28733.1). The synthetic expression vector pJExpress411 with Kanamycin resistance and high copy origin had been previously modified by the insertion of an RBS insulator (BCD2) cassette from (Mutalik et al., 2013). Kinase domains were fused to a mCitrine fluorescent protein (Griesbeck et al., 2001) and a Twin-Strep (Schmidt et al., 2013) purification tag through Gibson Assembly (Gibson, 2011) or Ligase Cycling Reaction (LCR) (Kok et al., 2014).

Expression screening

25 µl BL21 (DE3) chemically competent cells (lab made) were transformed with 100 ng of each plasmid (200 ng total when co-expressed with phosphatase). Incubation was as follows: 30 min on ice, 30 s at 42°C (water bath) and 5 min on ice; followed addition of 1 mL S.O.C. medium and recovery in 5 ml Nalgene tubes for 1 h at 37°C and 250 rpm shaking. Cells were concentrated by centrifugation at room temperature for 1 min at maximum speed (Eppendorf 5804R), resuspended in 200 µl of S.O.C. media of which 150 µl were plated on LB Agar plates containing 1% glucose and the respective antibiotic (Kanamycin 50 µg/ml, Carbenicillin 100

µg/ml, Ampicillin 100 µg/ml or Spectinomycin 50 µg/ml) and incubated overnight at 37°C. A starter culture was prepared in sterile 24-deep well plates with 1 ml of 2xYT media containing 1% glucose and the respective antibiotic. Three colonies of each construct were inoculated to grow overnight (~16 h) at 37°C and 250 rpm shaking. The starter cultures were washed by centrifugation, re-dissolved in 2xYT media (containing glucose and antibiotics as before) and used to inoculate a 4 ml production culture at 1:100 dilution (2xYT, 1% glucose, appropriate antibiotic) in sterile 24-deep-well plates. Incubation was performed at 37°C and 250 rpm shaking until OD_{600} of 0.5. Isopropyl ß-D-1 thiogalactopyranoside (IPTG) at high (0.5 mM) and low (0.05 mM) concentrations was added to induce protein expression for 18 h at 20°C or 3 h at 37°C. After a final measurement of OD_{600} and fluorescence, cultures were harvested (10 min at 1500 g, 20°C) and resuspended in normalizing buffer (20 mM HEPES, pH 8, 0.5% Triton-X, 1 mM DTT) with appropriate volume to adjust to an equal cell density. 400 µl of normalized cell suspension were mixed with 250 µl of lysis buffer (20 mM HEPES, pH 8, 0.5% Triton-X, 1 mM DTT, 25 U/mL Benzonase HC, ~1 mg/mL lysozyme), followed by 5 min of water bath sonication (30 s ice/30 s sonication) and 30 min shaking at 200 rpm at 4°C. Cell debris was removed by 20-30 min centrifugation. The supernatant was transferred to a black 96-well plate with flat bottom for fluorescence reading (excitation 516 nm, emission 528 nm).

Large-scale expression and purification

Starter cultures were inoculated from a single colony each and grown overnight at 37°C and 250 rpm shaking in 250 mL flasks with 100 mL of 2xYT media containing 1% glucose and the appropriate antibiotic. Production culture was inoculated at a 1:100 dilution in 1 L of 2xYT (1% glucose and antibiotics) and incubated at 37°C, 250 rpm until OD_{600} reached 0.5-0.6. Protein expression was induced with IPTG at a final concentration of 0.05 mM for 18 h at 20°C and 250 rpm. Cell pellets were harvested (15 min at 7,000 g) and washed with chilled PBS (pH 8) and stored at -80°C. Pellets were thawed and dissolved in 1 mL lysis & binding buffer per g of pellet (Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, Compete protease inhibitor (EDTA-free, Roche, 1 tablet/30 mL), 0.1 mM PMSF, 25 U/mL Benzonase HC (Milipore), 0.05% Triton, 10% glycerol), lysozyme (Sigma-Aldrich) was added at ~1 mg/ml, followed by sonication (2.5 min, 35% amplitude, 1 s *on* / 3 s *off*). The lysate was clarified by high speed centrifugation at 27,000 g for 1 h at 4°C.

Purification was performed on an ÄKTA HPLC (GE Healthcare) using a StrepTrap HP affinity column (GE Healthcare) equilibrated in binding buffer (100 mM Tris-HCl, pH 8, 150 mM

NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol) and recovered with elution buffer (2.5 mM D-desthiobiotin (Sigma) in binding buffer). PTP1B co-expressed samples were, in addition, filtrated through a GSTrap HP column (GE Healthcare) equilibrated to PBS, pH 7, 10% glycerol and 2 mM DTT. Size exclusion chromatography was performed on a Superdex S200 column (GE Healthcare) with gel filtration buffer (20 mM HEPES, pH 7.4, 300 mM NaCl, 10% glycerol, 2 mM DTT). Peak fractions were pooled and concentrated using Amicon Ultra 30K concentrators (Milipore) and aliquots were snap frozen in liquid nitrogen and stored at -80°C.

Kinase and phosphatase activity assay

Enzyme activity was measured in 5 µl reactions using the Z'LYTE kinase assay kit (ThermoFisher, with Tyrosine 2 peptide for Lyn and Src or Tyrosine 7 peptide for FAK). Each reaction contained 2.5 µl of purified kinase sample at different concentrations (50 nM, 500 nM, 2500 nM) and 2.5 µl of the respective 2x buffer-peptide master mix. Two types of master mixes were prepared: 1) Using a phosphorylated P(+) substrate peptide (as kinase positive control or as substrate peptide for de-phosphorylation reactions); 2) Non-phosphorylated (P-) substrate peptide (as kinase negative control, phosphatase activity positive control or kinase substrate peptide). Both master mixes were supplemented so that the final kinase reaction contained 0.5 mM DTT, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 100 µM ATP, 2 µM peptide substrate and 1x phosphatase inhibitor cocktail (Cell Signaling technology). Kinase reactions were incubated for 3 h at 37°C with 300 rpm shaking. 2.5 µl of the diluted development reagent were added, followed by 1 h incubation at room temperature and 300 rpm. The development (protease) reaction was stopped by the addition of 2.5 µl Stop Reagent. Fluorescence was read on a Tecan M1000 plate reader (Coumarin: excitation 400 nm, emission 445 nm; Fluorescein: excitation 400 nm, emission 520 nm). In the positive and negative control reactions, kinase sample was replaced by an equal volume of water (2.5 µl).