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Use of Phage Display to Generate Conformation-Sensor Recombinant Antibodies

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Abstract

We describe a phage display approach that we have previously used to generate conformation-sensor antibodies that recognize specifically and stabilize the oxidized, inactive conformation of protein tyrosine phosphatase 1B (PTP1B). We use a solution-based panning and screening strategy conducted in the presence of reduced active PTP1B, which enriches antibodies to epitopes unique to the oxidized form, while excluding antibodies that recognize epitopes common to oxidized and reduced forms of PTP1B. This strategy avoids conventional solid-phase immobilization, with its inherent potential for denaturation of the antigen. In addition, a functional screening strategy selects scFvs directly for their capacity for both specific binding and stabilization of the target enzyme in its inactive conformation. These conformation-specific scFvs illustrate that stabilization of oxidized PTP1B is an effective strategy to inhibit PTP1B function; it is possible that this approach may be applicable to the PTP family as a whole. Using this protocol, isolation and characterization of specific scFvs from immune responsive animals should take ~6 weeks.

Keywords

Conformation-sensor antibody; phage display; PTP1B oxidation; in vivo biotinylation; in-solution panning

INTRODUCTION

The term antibody phage display library refers to a collection of phage particles that display recombinant antibody fragments fused to surface proteins^{1–5}. Antibody phage display is a powerful and effective technique for generation and selection of specific antibodies from a large and diverse collection of functional antibodies in the library⁶. The antibody fragment-expressing genes can be produced from a naive source⁷, or derived after immunization², or they can be produced by introducing random mutations *in vitro* in one or more complementarity determining regions (CDRs)³. Recombinant antibodies presented at the surface of bacteriophage can be selected from combinatorial libraries under extensively controlled conditions^{8,9}. This *in vitro* process allows selection of antibodies to highly

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Author Contributions

A.H. and N.K.T. designed the experiments and analyzed the data. A.H. performed the experiments. A.H. and N.K.T. wrote the manuscript. NKT directed the study and obtained grant support. The authors read and approved the final version of the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

conserved antigens. In comparison with traditional approaches of generating antibodies from animals this has the major advantage of preserving the natural conformation of the antigen of interest to allow the generation of conformation-specific antibodies by successive rounds of selection or panning^{10, 11}. Among several different methods for selecting antibody fragments, including ribosomal, bacterial or yeast display, antibody phage display has proven to be a robust, versatile and extensively applied method¹².

For selection of antibodies for a target antigen two functional antigen-binding fragments, designated Fab and scFv, can be displayed on phage¹¹. The Fab (fragment antigen-binding) is composed of one constant and one variable domain of each of the heavy and light chains, whereas the smaller Fv (fragment variable) is composed of the variable light (V_L) and variable heavy (V_H) regions. A single chain variable fragment (scFv) is generated by joining the two variable regions with a flexible peptide linker (Figure 1) and expressed as a recombinant polypeptide chain fused to the surface protein of phage particles¹³. The scFvs can be expressed as single polypeptides, which retain the binding specificity of functional antibodies. Use of scFv offers several advantages over Fab. The smaller insert size in the scFv libraries render it relatively more stable than the Fab libraries¹⁴. The Fab fragments are twice the size of the scFvs and require more extensive assembly and folding in the bacterial expression system¹⁵. Moreover, the scFv expression generally has a less toxic effect on the bacteria than the larger Fab fragments. As a result the functional scFvs result in a significantly better yield in *E. coli*⁵. Generation and screening of specific functional antibodies using the scFv library also offers practical benefits. Use of short, single chain fragments makes the construction of a large and diverse library easier¹⁵. It is also more convenient to generate higher affinity mutants of scFv by site-directed mutagenesis or antibody engineering¹⁶. One of the major advantages of using this approach is that the candidate antibodies can be expressed inside mammalian cells as functional intracellular antibodies or “intrabodies”^{17–25}, and used as probes of the function, and post-translational modification, of intracellular targets. This protocol was developed to produce scFvs that recognize selectively the oxidized conformation of the protein tyrosine phosphatase PTP1B, which is generated in vivo in a wide variety of signaling contexts, and to test their potential as tools to modify the signaling function of the enzyme.

PTP1B is the prototypic member of the protein tyrosine phosphatase (PTP) family²⁶ and plays a pivotal role in regulating insulin and leptin signaling^{27, 28}. PTP1B contains a signature catalytic motif (the PTP loop), [I/V]HCXAGXXR[S/T]G, which is conserved among all PTPs²⁹. Cys215 and Arg221 within this motif are indispensable for phosphatase activity²⁹. Based on its important role in regulating insulin and leptin signaling, considerable interest has grown in harnessing PTP1B as a therapeutic target, particularly for treatment of diabetes and obesity^{30, 31}. The highly charged catalytic core of the reduced active form of PTP1B, however, has been a major obstacle to developing active site-directed inhibitors of this enzyme that have appropriate drug development potential. Moreover, selectivity of inhibitors over closely related PTPs has been a challenge because of the highly conserved nature of the active site across the PTP family. For example, the catalytic domain of T-cell protein tyrosine phosphatase (TCPTP) displays ~75% sequence identity to PTP1B and the tertiary structures of these two enzymes overlap closely^{32, 33}; in fact most of the potent catalytic site-directed PTP1B inhibitors also show some degree of inhibition of TCPTP activity³⁴. Consequently, novel approaches are required to generate inhibitors of the PTP family that would be acceptable for drug development.

PTPs are oxidized and inactivated by reactive oxygen species (ROS) produced in response to a variety of physiological stimuli, including insulin^{35–38}. Analysis by X ray crystallography revealed that mild oxidation of the active site cysteine of PTP1B generates a 5-atom cyclic sulphenyl-amide species, in which the sulphur atom of the active site cysteine

is covalently linked to the main-chain nitrogen of the adjacent serine residue^{39, 40}. In this reversibly oxidized form (PTP1B-OX), elements of the catalytic cleft of PTP1B, including the PTP loop and Tyr46 of the pTyr loop, are presented on the protein surface (Figure 1), which inhibits substrate binding and renders the enzyme inactive. The profound conformational change at the active site that is induced by reversible oxidation presents new, unique binding surfaces. If this transiently oxidized form of PTP1B can be stabilized, locking the enzyme in an inactive conformation, then this may provide an alternative approach to manipulate the function of the enzyme under pathophysiological conditions. Our goal was to test this hypothesis by generating scFvs, directed against oxidation-specific epitopes of PTP1B, which could be expressed in cells as intrabodies that would stabilize the oxidized conformation of PTP1B, thereby inhibiting its phosphatase function and promoting insulin signaling. This approach of inhibiting PTPs through stabilization of the inactive, oxidized conformation may represent a new way of inhibiting other redox sensitive PTPs selectively for pathophysiological intervention.

The isolation of specific antibodies against the oxidized conformation of PTP1B would be difficult by conventional antibody production techniques. Phage-display allows an easy and flexible process for selection of specific antibodies to defined epitopes presented on isolated, purified antigens. In most cases, the antigen of interest is presented to the antibody phage library on solid surfaces as an immobilized bait; however, immobilization limits the enrichment and selection of conformation-specific antibodies because the conformation of the epitope can be compromised, or improperly exposed⁴¹. An appropriate panning and screening method, which preserves a specific conformation of the antigen in conditions under which the epitope is properly presented, without structural alteration, is the main prerequisite for a successful selection strategy. Nizak et al. reported the generation of scFvs specific to the GTP-bound form of the GTPase Rab6 by performing selections against a GTP-locked mutant²¹. Covalent modification with a small molecule inhibitor, to lock the target antigen in a particular conformation for selection purposes, has also been reported⁴². Eisenhardt et al. described a subtractive panning strategy to select conformation-specific antibodies against cell surface targets⁴³. PTP1B, however, is a tail-anchored protein, in which the C-terminus targets the protein to the ER membrane with the catalytic domain exposed to the cytoplasm⁴⁴. Furthermore, endogenous PTP1B undergoes various degrees of oxidation, depending on signaling cues. Consequently, screening by targeting endogenous PTP1B directly is difficult. Therefore, we designed a solution-based, *in vitro* selection strategy and subsequent enzyme activity-based screening method that was crucial for preserving specific interactions between the candidate scFvs and a particular conformation of the antigen. We used this *in vitro* technique with several empirically optimized modifications to enrich and isolate candidate scFvs to PTP1B-OX systematically.

A major challenge to producing antibodies that are selective for particular conformations of a protein is to have homogenous preparations of the target antigen displaying that particular conformation in a stable manner. The crystal structures of the doubly mutated form of PTP1B (PTP1B-C215A/S216A, termed PTP1B-CASA) and the reversibly oxidized form are identical⁴⁵. Consequently, this PTP1B-CASA mutant captures the conformation of the oxidized enzyme in a stable manner, generating a homogeneous preparation of the antigen. The solution-based panning strategy, conducted in the presence of up to 50-fold molar excess of the reduced, wild type enzyme, ensured that antibodies recognizing specific epitopes on PTP1B-OX were enriched, whereas those that bound common epitopes on both conformations of the protein were depleted. To enrich specific scFvs from the phage display library we used biotinylated PTP1B-CASA and streptavidin bead capture. Although similar approaches have been reported previously for isolating scFvs^{21, 46, 47}, we adopted an *in vivo* biotinylation method (Box 1). We fused a sequence motif to the N-terminus of the target antigen (PTP1B-CASA), which allowed addition of a biotin moiety specifically to a single

Lys residue found only in the tag, leaving the rest of the protein unmodified⁴⁸. This was important because biotinylation of PTP1B by this method did not alter enzyme activity, whereas modification by chemical biotinylation was inhibitory. This suggests that the specific addition of biotin to the N-terminal tag did not cause any unwanted structural change in PTP1B, and that oxidation-specific epitope(s) in the biotinylated PTP1B-CASA protein were preserved.

This protocol provides a thorough, step-by-step description of the panning and screening strategies for isolating scFvs that bind specifically to the oxidized conformation of PTP1B. Specific scFvs were selected using an in-solution screen that exploited an *in vitro* phosphatase assay (Box 2) to measure reactivation of PTP1B-OX that had been reversibly oxidized by H₂O₂ in vitro. Through our screening method we isolated scFvs that not only bound to PTP1B-OX, but also stabilized the inactive conformation and inhibited its reactivation by reducing agent. This novel approach to inhibition of PTP1B function suggests that small molecules that mimic the stabilizing effects of the scFvs may have therapeutic potential. Oxidation of PTPs is emerging as a regulatory mechanism for many different cellular pathways⁴⁹. In addition to PTP1B, specific examples include the oxidation of SHP2 in PDGF signaling³⁵, TC-PTP in insulin signaling³⁸ and multiple PTPs in B-cell receptor signaling^{50, 51}. In addition, the oxidation of several receptor PTPs including, PTP α and LAR⁵², dual-specificity PTPs (e.g. PTEN and MKP-1^{52–54}) have been reported. In fact, a similar cyclic sulphenyl amide modification has been observed in the active site of oxidized PTP α ⁵⁵. Considering the key regulatory roles of these PTPs, the development of conformation-sensor antibodies that can recognize and stabilize the oxidized inactive forms of these enzymes will generate important tools with which to investigate the regulation of cell signaling. The activity of many other biologically important regulatory proteins also depends on conformational switches^{36, 56, 57}. Overall, our protocol describes an approach that applies not only to the PTP family as a whole, but may also represent a general scheme to select and test the ability of scFvs to recognize and stabilize biologically relevant protein conformers so as to alter a particular biological function or pathway.

Experimental design

Construction of Antibody Phage Display Library

Particular attention should be paid to the preparation of antigen. It is very important to generate a preparation in which the required conformation is adopted in a stable manner. In our case, this was facilitated by detailed structural analysis, which revealed that the PTP1B-CASA double mutant was locked in the same conformation as oxidized PTP1B. This understanding of the structural consequences of oxidation was a key element of success. The 37 kDa form of wild type PTP1B or the PTP1BCASA mutant (comprising residues 1–321 without any epitope tag) were expressed in *E. coli* and purified to homogeneity by following the method described in⁵⁸. This form of PTP1B comprises the catalytic domain, however, in the cell the native enzyme is expressed as a longer protein with a non-catalytic C-terminal segment that is challenging to express in homogeneous form due to sensitivity to proteolysis. This detailed understanding of the properties of the target antigen permitted us to focus on a form of the enzyme that is stable, readily purified and preserves the important conformational changes we wished to study.

A phage display library displaying single chain variable fragments (scFvs) fused to phage surface protein pIII was generated from immune genes collected from spleen and bone marrow of chickens immunized with PTP1B-CASA. One can also produce scFv library from naive source (Hoogenboom and Winter, 1992), or they can be produced by introducing random mutations *in vitro* in one or more complementarity determining regions (CDRs) (Griffiths et al., 1994). Generation of antibody phage display libraries from animals already

immunized with the antigen is advantageous because diversity for the antigen is already created *in vivo* by the immune system^{2, 59}. Many antigens of mammalian origin are not present or less conserved in avian species, such as chickens, which are a useful source for producing a high level of immune genes against the antigen of interest. In addition, chickens generally produce a higher level of immune response than mice and rabbits^{60, 61}. Detailed protocols for construction of an scFv library from immunized chickens can be found in^{60, 62}.

A diverse library is an important prerequisite for successful isolation of specific scFvs. We constructed two different types of scFvs, one with a short linker sequence (GQSSRSS) and one that was longer (GQSSRSSSGGGSGGGG), and mixed them together to add diversity to the library. Diversity can be further increased during the generation of scFvs, by varying the order of the variable domains at either the N-terminus or at the C-terminus (Bird et al., 1988; Huston et al., 1988) or by changing the length of the polypeptide linker between the two variable domains (Huston and Haber, 1996). Linkers having different lengths may lead to different degrees of oligomerization of the generated scFvs. Reports have shown that scFvs with the shorter linkers tend to form dimers as compared to the longer linkers, which tend to form monomers⁶³. After the panning steps we mostly observed the enrichment of scFvs with the shorter linker. Only one clone with the longer linker was found, but when purified as soluble scFv from bacteria it did not demonstrate any binding or stabilizing effect on PTP1B-OX.

Combining replicates of the PCR amplification and ligation reactions during the construction of the library can improve the scFv diversity. Also to maintain the initial diversity of the library, large volumes of culture can be used to inoculate high number of infected cells during the selection process. One concern about maintaining the original diversity of the library is that each round of amplification can result in a reduction of diversity in the scFv repertoire because of potential outgrowth of fast growing clones. It is, therefore, important to use a library that has been freshly amplified from the original library before starting the selection process. Libraries that have undergone several rounds of amplification should be reassessed for the presence of full-length scFv inserts and the diversity by sequencing randomly picked clones.

Expression and Purification of *in vivo* Biotinylated PTP1B (see Box 1 and Figure 2)

For use of the antigen of interest in the in-solution panning (Steps 1–20) it is essential to purify biotinylated target antigen in homogeneity. Recombinant PTP1B can be readily expressed in BL21 *E. coli*; therefore, this expression system was used to co-express PTP1B and biotin ligase. If another bacterial system was to be used, the conditions for co-expression of both proteins, biotinylation efficiency and purification steps would have to be optimized accordingly. Initially, we used a chemical biotinylation approach, which added biotin moieties to free amines non-specifically. This method caused significant inhibition of PTP1B activity, probably by modifying the chemical and structural integrity of the active site or other important sites required for activity. To isolate conformation-specific scFvs, it is important to avoid modifications that may affect structural integrity. Therefore, we adopted an *in vivo* approach to biotinylate PTP1BCASA specifically using a biotinylation tag fused to the N-terminus of the protein. Biotin ligase specifically recognizes this 15-residue peptide tag (GLNDIFEAKIEWHE) and in presence of ATP, attaches biotin to the lysine residue in this sequence⁴⁸. The pBirAcm plasmid, which was isolated from DNase-deficient K-12 *E. coli* strain (AVB99, Avidity), is an engineered pACYC184 plasmid (ColEI compatible) that harbors an IPTG-inducible birA gene to over-express biotin ligase, which adds biotin specifically to the lysine residue in the biotinylation tag. Addition of biotin in the growth media during the time of induction ensures biotinylation of the tagged protein. If *in vivo* biotinylation of the target antigen is difficult, one can biotinylate the target antigen

specifically by purifying the protein with the biotinylation tag and then using commercially available biotin ligase (Avidity) to add biotin *in vitro*.

In-Solution Panning (Steps 1–20 and Figure 3)

Panning or selection of specific scFvs can be performed in several ways. Immobilization of the antigen on a solid surface is perhaps most common; however, other methods such as use of whole cells, tissue sections and *in vivo* panning have been reported⁶⁴. Immobilization causes random attachment of the antigen onto the plastic surface without any uniform directional orientation and may lead to partial denaturation⁴¹; consequently, the conformational difference at the active site between the reduced and oxidized form of proteins may not be preserved under these conditions. In some protocols, the antigen is attached to plastic by initially treating the surface with high pH coating buffer. This elevated pH may also cause denaturation of the protein resulting in improper presentation of the conformational epitope(s) of interest.

In our protocol, selection of specific scFvs is performed in solution, without structural alteration of the target antigen due to chemical modification or immobilization. Biotinylation of PTP1B-CASA on an N-terminal tag did not cause non-specific modification in the catalytic domain of the enzyme and did not affect the function of the wild type enzyme or its reactivation status after oxidation. Maintaining the structural integrity of the tagged antigen throughout the panning steps is important to permit isolation of the conformation-sensor scFvs from solution. Furthermore, we modified this solution-based panning by simultaneous addition of molar excess of reduced active enzyme (PTP1B-WT) together with the biotinylated target antigen (PTP1BCASA) to enrich for scFvs that are specific for the oxidized conformation. Wild type PTP1B should be stored in the presence of reducing agent to avoid oxidation and inactivation in the storage buffer. Repeated freeze-thaw cycles should also be avoided for both the wild-type and mutant proteins. From this in-solution mixture, scFv-displaying phage particles bound to the biotinylated antigen (CASA mutant) were captured using streptavidin-coated magnetic beads. Amplified phage from the first round of selection were applied in the second round of panning, and this process was repeated for five rounds of panning following similar selection conditions. Complete in-solution panning comprises phage pre-adsorption, addition of both reduced wild type PTP1B and the biotinylated CASA mutant, capture of the scFv-phage-antigen complex with Streptavidin coated magnetic beads, elution of the phage particles and phage amplification. We used acidic buffer conditions to elute the scFv-expressing phage particles from the antigen-phage (scFv)-magnetic bead complex. By changing the mode of elution (acid vs. base) different subsets of scFv population may be obtained and characterized.

In any selection, special procedures should be designed to minimize the enrichment of nonspecific sequences. Binding buffer should be supplemented with BSA to reduce nonspecific binding of scFvs to the magnetic beads and the plastic surface of the tubes. Appropriate wash steps are important for efficient enrichment of the specific scFvs. If too stringent, washes may remove not only nonspecific scFvs, but also specific scFvs with low affinity to the target. The number of panning steps is a vital factor for selection and enrichment of specific scFvs. This can be determined empirically. In most procedures 3–4 rounds of panning are performed and then the sequence of the output scFv can be verified to determine the enrichment of the library. The frequency of unique scFvs present after each panning step provides an indication of diversity and enrichment.

Validation of the Library Quality and the In-Solution Panning

After each round of panning, scFvs should be sequenced for quality control analysis of the library and to validate the progress of the panning experiment by assessing the initial

diversity and subsequent enrichment of specific scFvs. It is important to verify that the scFv inserts are antibody fragments derived from the species of interest. It has to be ensured that full-length scFvs are present in the library without unwanted cloning errors, such as deletion, premature stop codons or no insert at all! Alternatively, scFv sequence variations can be verified by rapid and inexpensive analysis of the restriction digestion patterns by *BstOI* endonuclease and agarose gel electrophoresis. This method, however, is limited by the resolving power of the agarose gel.

In-Solution Functional Screening (see Figure 4)

The yield of purified scFvs depends on expression system, purification conditions and the variable region of the individual scFv. Expression level for phagemid (i.e., pComb3XSS) is generally high enough for using the purified scFvs for direct screening. If required, higher scFv yields can be obtained through subcloning into T7-based vectors (i.e., the pET expression vectors from Novagen) along with BL21(DE3) expression system⁶⁵. Some scFvs may demonstrate increased aggregation or precipitation and formation of inclusion bodies and this can be minimized by using specially engineered *E. coli* strains for expression. These strains have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and greatly enhance folding of the scFvs in the *E. coli* cytoplasm⁶⁶. Alternatively, scFvs expressed using the pComb3XSS vector can be extracted from the periplasmic space by removing the outer membrane of *E. coli* with osmotic shock buffer (0.5 M sucrose in PBS/TBS). This generally yields a relatively pure preparation of scFvs. The purified scFvs should be stored in aliquots at -20 or -80 °C and repeated freeze-thaw cycles should be avoided. For better stability it is recommended to store purified scFv at a concentration of 1 mg/ml or higher. If getting high concentration of scFv is not possible the protein can be concentrated or BSA (10 mg/ml) can be added before freezing.

Screening strategies based solely on the ability of an scFv to bind to the antigen would not provide information on the ability of that scFv to stabilize PTP1B-OX and inhibit its reactivation. To analyze systematically individual scFvs from enriched pools of phage particles after the in-solution panning, we employed a phosphatase assay in solution (Box 2) to detect whether any scFvs inhibited reactivation of oxidized PTP1B by reducing agent. Consequently, establishing conditions under which PTP1B is reversibly oxidized by H_2O_2 in solution is of vital importance for screening for such scFvs. It would be anticipated that conformation sensor scFvs that recognize the oxidized form would not exhibit direct inhibitory effects on the phosphatase activity of the active, reduced form of PTP1B. Therefore, it is an essential control to test this directly in assays of phosphatase activity. The scFvs can be further characterized by binding experiments with the reduced and oxidized form of the target protein. Such binding experiments can be performed by Ni-NTA or HA pull down, exploiting the C-terminal 6His or HA tags in the scFvs, respectively, and subsequent immunoblotting of the target protein. It is also useful to determine the binding constants for the interaction between the selected scFvs and the antigen by Surface Plasmon Resonance (SPR). Selected individual scFvs identified on the basis of this in vitro strategy can be further validated by testing their ability to stabilize a particular conformation of the target protein in mammalian cells and to modify the cellular function of the protein⁴⁰.

MATERIALS

REAGENTS

1,4-Dithiothreitol (DTT), ultrapure (Roche, cat. no. 03117006001)

CRITICAL: DTT is unstable in water; it is best always to prepare a fresh stock solution shortly before use.

3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid, MOPS (Sigma, cat. no. M1254)

Ampicillin sodium salt (Sigma, cat. no. A0166)

AmpliTaQ Gold DNA polymerase (Applied Biosystems, cat. no. 4338857)

Anion exchange column (HiTrap Q FF, GE Health Sciences, cat no. 17-5053-01)

Benzamidine hydrochloride hydrate (Sigma, cat. no. B6506)

Blue/orange loading dye (Promega, cat. no. 1881)

Bradford protein assay dye reagent concentrate (Bio-Rad, cat. no. 500-0006)

! CAUTION Toxic. Contains methanol.

BSA (Roche, cat. no. 03117057001)

Carbenicillin disodium salt (Sigma, cat. no. C1389)

Chloramphenicol (Sigma, cat. no. C1919)

Complete protease inhibitor cocktail, EDTA free (Roche, cat. no. 11873580001)

CRITICAL: For using in buffers for purification or pull down with Ni-NTA matrix, the protease inhibitor must be EDTA-free, as EDTA can strip nickel from the nickel-affinity support.

D-Biotin (MP Biomedicals, cat. no. 101023)

DNA ladder 1 kb Plus (Invitrogen, cat. no. 10787-018)

Ethanol (Pharmco, cat. no. 111ACS200)

! CAUTION Flammable.

Ethidium bromide (Sigma, cat. no. E1510)

! CAUTION Carcinogenic. Wear protective gear, avoid contact and handle with care.

Ethylenediaminetetraacetic acid (EDTA) (Sigma, cat. no. EDS)

EZ-Link Sulfo-NHS-LC Biotinylation Kit (Thermo Scientific, cat. no. 21425)

Glucose (Sigma, cat. no. G8270)

Glycerol (J.T. Baker, cat. no. M778-09)

Glycine (Sigma, cat. No. G7126)

GST-FER kinase: See reference⁶⁷ for preparation of GST-FER kinase.

H₂O₂ (Sigma, cat. no. H1009)

! CAUTION Corrosive, oxidizing agent, handle with care, avoid contact with skin and face.

HABA (2-[4'-hydroxyazobenzene]-benzoic acid) (Sigma, cat. no. H-5126)

HEPES (Sigma, cat. no. H3375)

Imidazole (Sigma, cat. no. 12399)

Immobilized Monomeric Avidin resin (Thermo Scientific, cat. no. 20267)

Iso-propanol (Sigma, cat. no. I9516)

Isopropyl- β -D-thiogalactopyranoside (IPTG) (Roche, cat. No. 11411446001)

Kanamycin (Sigma, cat. no. K1876)

Luria Bertani (LB) agar (AMRESCO, cat. no. J637)

Lysozyme (Sigma, cat.no. L6876)

CRITICAL: Store powder at -20°C and dissolve at 10 mg ml^{-1} in PBS just before use.

Magnesium chloride hexahydrate (Fisher, cat. no. M33)

Manganese(II) chloride tetrahydrate (Sigma, cat. no. 221279)

NiCl_2 (Sigma, cat. no. N6136)

Ni-NTA Agarose (Qiagen, cat. no. 30210)

PCR nucleotide mix (Roche, cat. no. 04638956001)

PEG 8000 (Sigma, cat. no. P2139)

Phenylmethanesulfonyl fluoride (PMSF) (Sigma, cat. no. P7626)

QIAprep spin plasmid preparation kit (Qiagen, cat. no. 27104)

QIAquick gel extraction kit (Qiagen, cat. no. 28704)

Recombinant PTP1B (1–321): See reference⁵⁸ for expression and purification.

Recombinant *in vivo* biotinylated PTP1B (1–321)-WT (Box 1)

Recombinant *in vivo* biotinylated PTP1B (1–321)-CASA (Box 1)

CRITICAL: It is essential to use the *in vivo* biotinylated PTP1B-CASA mutant for the panning experiment as biotinylation by chemical methods adds biotin non-specifically. Most likely this chemical modification causes structural modifications, which are manifested by a reduction in the enzyme activity of chemically biotinylated PTP1B.

Reduced carboxamidomethylated and maleylated lysozyme (RCML): See reference^{67–69} for preparation of RCML.

Restore stripping buffer (Thermo Scientific, cat. no. 21059)

SeaKem GTG agarose (Lonza, cat. no. 50074)

Sodium acetate (Sigma, cat. no. S7545)

Sodium azide (Sigma, cat. no. S2002)

! CAUTION Toxic. Avoid contact.

Sodium chloride, NaCl (Amresco, cat. no. X190)

Sodium phosphate dibasic, Na₂HPO₄ (Sigma, cat. no. S5136)

Sodium phosphate monobasic, NaH₂PO₄ (Amresco, cat. no. 0571)

Streptavidin coated magnetic bead (Dynabeads M-280, Invitrogen, cat. no. 112-05D)

SuperScript preamplification system for first strand cDNA synthesis (Life Technologies, cat. no. 18089-011)

T4 DNA ligase (Invitrogen, cat. no. 15224025)

Tetracycline (Sigma, cat. no. T3383)

Trichloroacetic acid, TCA (JT Baker, cat. no. 0414-01)

Tris (AMRESCO, cat. no. 0497)

Tris(2-carboxyethyl)phosphine (TCEP) (Thermo Scientific, cat. no. 77720)

Tris-HCl (Sigma, cat. no. T3253)

Triton-X100 (Sigma, cat. no. T9284)

Tween-20 (Sigma, cat. no. P1379)

VCSM13 interferon-resistant helper phage (Stratagene, cat. no. 200251)

Yeast extract (Sigma, cat. no. Y1625)

Zeba desalting columns (Thermo Scientific, cat. no. 89882)

Restriction endonucleases—NcoI (New England Biolabs, cat. no. R0193S),

SfiI (Roche, cat. no. 11288059001)

Bacterial Strains—XL-1 blue *E. coli* (Stratagene, cat. no. 200228)

CRITICAL: Grow in LB media containing 30 µg/ml tetracycline.

TOP10F' *E. coli* (Invitrogen, cat. no. C3030-03)

BL21 *E. coli* (Invitrogen, cat. no. C6000-03)

K-12 *E. coli* strain (Avidity, cat. no. AVB99)

CRITICAL: Grow in LB media containing chloramphenicol at 10 µg/ml concentration

Plasmid Vectors—pET19b vector (EMD Biosciences, cat. no. 69677-3)

pBirAcm plasmid, an engineered pACYC184 plasmid (ColEI compatible) with an IPTG inducible birA gene to over-express biotin ligase, isolated from K-12 *E. coli* strain (Avidity, cat. no. AVB99)

pComb3XSS phagemid vector (from Dr. Carlos Barbas laboratory; see GeneBank accession no. AF268281 for complete sequence of the phagemid cloning vector pComb3X).

Antibodies—Anti-biotin-HRP antibody (Cell Signaling, cat. no. 7075S)

Anti-HA monoclonal antibody, Clone 3F10, conjugated to Agarose beads (Roche, cat. no. 11815016001)

Anti-HA-HRP, clone 12CA5 (Roche, cat. no. 11667475001)

Anti-HA-Peroxidase, high affinity, clone 3F10 (Roche, cat. no. 12013819001)

Anti-His mouse monoclonal antibody (Genescript, cat. no. A00186)

Anti-PTP1B monoclonal antibody, clone FG6 (prepared in the Tonks' Lab)

Anti-PTP1B antibody, rabbit polyclonal antibody, clone H-135 (Santa Cruz, cat. no. sc-14021]

EQUIPMENT

Amicon Ultra-15 Centrifugal Filter Unit (Millipore, cat. no. UFC901008)

Amicon Ultra-4 Centrifugal Filter Unit (Millipore, cat. no. UFC801024)

Bottles, wide-mouth, 250 ml (Thermo Scientific, cat. no. 2105-0008)

Bottles, wide-mouth, 60 ml (Thermo Scientific, cat. no. 2105-0002)

Centrifuge (Beckman, Allegra 6)

Centrifuge (Beckman, Avanti J-26 XP)

Centrifuge (Beckman, Avanti J-E)

Centrifuge (Beckman, J6-M1)

Centrifuge (Eppendorf, 5415D)

Centrifuge (Eppendorf, 5417C)

Centrifuge (Eppendorf, 5424)

Dialysis Cassettes, 10K MWCO, Slide-A-Lyzer (Thermo Scientific, cat. no. 66380)

Disposable chromatography columns (Bio-Rad, cat. no. 731-1550)

Electroporation device (Bio-Rad, Gene Pulser Xcell)

Falcon conical tubes, 225 ml (BD, cat. no. 352075)

Falcon tubes, 15 ml (Corning, cat. no. 430766)

Falcon tubes, 50 ml (Corning, cat. no. 430290)

Filter, 0.2 μ m (Pall Corporation, cat. no. PN 4192)

Filter, 0.45 μ m (Pall Corporation, cat. no. PN 4184)

Glass beads (Fisher Scientific, cat. no. 11-312A)

Incubator 30 °C

Incubator at 5% CO₂ and 37 °C

Microcentrifuge tubes, 1.5mL, siliconized, sterile, individually wrapped (VWR, cat. no. 22179-006)

Scintillation counter

Shaker incubators at 30°C

Shaker incubators at 37°C

Sonifier (Branson 450)

SpectraMax 190 absorbance microplate reader (Molecular Devices)

Stationary incubators (37 °C)

Sterile Erlenmeyer flasks (250, 500, 1,000 and 2,500 ml)

UV lamp

Vacuum pump

Water baths

Computer programs—MultAlin (<http://multalin.toulouse.inra.fr;>)

REAGENT SETUP

Ampicillin Stock: 50 mg/ml in ddH₂O

Dissolve 2.5 g of ampicillin in 50 ml of ddH₂O. Filter through a 0.2- μ m disc filter and make 1-ml aliquots.

CRITICAL: Store at –20°C up to 1 year. Freshly dilute thawed aliquots into medium or agar.

Benzamidine hydrochloride: 1M in 100% ethanol

Dissolve 7.8 g in 50 ml ethanol. Make 1-ml aliquots and store at –20°C for 1 year.

CRITICAL: Unstable in water. Dilute thawed aliquot of the frozen stock just before use.

Bradford Reagent: Add 10 ml of Bradford reagent concentrate (5 $\times$) in 40 ml ddH₂O and mix.

Diluted reagent can be stored at room temperature for several weeks.

BSA Blocker Solution: 10% (wt/vol) BSA in TBS. Add 10 g of BSA in 100 ml of TBS, mix to dissolve completely. Filter through a 0.2 μ m filter unit to remove microbial contamination.

CRITICAL: Add 0.02% sodium azide and store at 4°C for several months.

Carbenicillin Stock: 100 mg/ml in ddH₂O

Dissolve 5 g of carbenicillin in 50 ml of ddH₂O. Filter through a 0.2- μ m disc filter and make 1-ml aliquots.

CRITICAL: Store at –20°C up to 1 year. Freshly dilute thawed aliquots into medium or agar.

Chloramphenicol stock solution: 34 mg/ml in 100 % ethanol

Dissolve 0.34 g of chloramphenicol in 10 ml of 100% ethanol and make 1-ml aliquots.

CRITICAL: Store the stock at –20°C up to 1 year. Freshly dilute aliquots into medium or agar.

EDTA Solution: 0.5 M, pH 8.0 in ddH₂O

Add 18.61 g EDTA (sodium salt) to 90 ml ddH₂O. Adjust pH to 8.0 and add ddH₂O to make the volume 100 ml. Filter sterilize using a 0.2 μ m filter and store at room temperature for years.

Glucose Solution: 20% (wt/vol) in ddH₂O

Dissolve 20 g of glucose in 100 ml of ddH₂O. Filter through a 0.2- μ m filter unit. Store at 4 °C for several months.

Glycine Elution Buffer: 0.2 M glycine-HCl, pH 2.2

Dissolve 3.75 g of glycine in 500 ml of ddH₂O. Adjust pH to 2.2 with HCl. Filter through a 0.2- μ m filter unit. Store at room temperature for several months.

! CAUTION HCl is Hazardous and corrosive; wear lab coat, gloves and use eye-protection.

Imidazole Solution: 1 M in ddH₂O

Add 17 g imidazole to 250 ml water. Dissolve thoroughly and filter with a 0.2 μ m filter unit. Store at room temperature for several months.

IPTG solution: 0.5 M in ddH₂O

Dissolve 6 g of IPTG in 50 ml of ddH₂O. Filter through a 0.2- μ m filter unit. Store at – 20 °C for up to 1 year.

CRITICAL: Freshly add the thawed stock to the medium. Avoid repeated freeze-thaw cycle.

Kanamycin Stock: 50 mg/ml in ddH₂O

Dissolve 2.5 g kanamycin sulfate in 50 mL ddH₂O and make 1-ml aliquots.

CRITICAL: Store at –20°C for several months. Freshly dilute thawed aliquots into medium or agar.

Luria Broth (LB): Dissolve 10 g of tryptone, 5 g of yeast extract and 10 g NaCl in 1 liter of ddH₂O. Autoclave and store at 4°C for several months.

MgSO₄ Solution: 1 M in ddH₂O

Dissolve 24.65 g of magnesium sulfate (heptahydrate) in 100 ml of deionized water, filter with 0.2 µm filter unit and store at room temperature for several months.

NaCl Solution: 5 M in ddH₂O

Dissolve 29.22 g of NaCl in 100 ml deionized water. Autoclave and store the solution at room temperature for several months.

Panning Neutralization Buffer: 1 M Tris base, pH 9.1

Dissolve 12.1 g Tris in 70 ml ddH₂O. Adjust pH to 9.1 with HCl. Adjust volume to 100 ml with ddH₂O and filter with 0.2 µm filter disc. Store at room temperature for several months.

PBS: 137 mM NaCl, 2.7 mM KCl, 12 mM Na₂HPO₄, 1.2 mM KH₂PO₄, pH 7.4

Dissolve 3.6 g Na₂HPO₄, 0.2 g KCl, 0.24 g KH₂PO₄ and 8 g NaCl in 1 liter of deionized water.

Adjust the pH to 7.4 and autoclave. Store the buffer at room temperature for several months.

PBST Buffer: 0.05% (vol/vol) Tween-20 in PBS

Supplement PBS with 0.05% (vol/vol) Tween-20. Filter-sterilize with 0.2 µm filter unit and store at room temperature for several months.

PEG/NaCl solution, 5×: 20% (wt/vol) polyethylene glycol-8000, 2.5 M NaCl in ddH₂O.

Dissolve 200 g PEG-8000 and 150 g of NaCl in ddH₂O. Autoclave and store at room temperature for several months.

CRITICAL: Dissolving PEG and NaCl into solution requires prolonged stirring. Make sure the components are dissolved before autoclaving.

Phosphatase Assay Buffer: 50 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% BSA (wt/vol) (with or without 5 mM TCEP)

Dissolve 1.19 g HEPES, 5.84 g NaCl and 1 g BSA in 900 ml ddH₂O. Adjust pH to 7.0 and add water to make 1L. Filter sterilize with 0.2 µm filter unit. Store at 4°C for several months. Add TCEP immediately before use.

PMSF: 100 mM in 100% isopropanol

Dissolve 0.87 g PMSF in 50 ml isopropanol. Store at –20°C for several months.

CRITICAL: PMSF quickly degrades in aqueous solution at neutral pH. Add to the buffer immediately before use. PMSF is extremely toxic, and preparation of solutions should be done very carefully.

Reduced Carboxamidomethylated and Maleylated Lysozyme (RCML): 20 mg/ml in ddH₂O

Dissolve 100 mg of RCML in 5 ml of ddH₂O. Make 1-ml aliquots and store at –20°C for several months.

SDS Sample Loading Buffer, 4×: Add 5 ml of 1M Tris-HCl, pH 6.8, 4 g of SDS, 20 ml of 100% glycerol, 5 ml of 0.5 M EDTA, 40 mg bromophenol blue and 13 ml H₂O and dissolve thoroughly. Store at –20°C for years. Add DTT (100 mM) final concentration immediately before use.

SOB Medium: 2% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 7.0, 10 mM MgCl₂, 10 mM MgSO₄

Add 20 g of tryptone, 5.0 g of yeast extract, 0.5 g of NaCl and 186 mg of KCl to 950 ml of ddH₂O. Mix to dissolve and adjust the pH to 7.0 with NaOH. Adjust volume to 980 ml with ddH₂O and autoclave. Add 10 ml of 1M MgCl₂ and 10 ml of 1M MgSO₄ (both filter sterilized) and store at room temperature for several months.

SOC Medium: 20 mM of glucose in SOB medium

Add 0.5 ml of 2 M glucose (filter-sterilized) to 50 ml of SOB and store at 4°C for several months.

Sodium Azide: 10% (wt/vol) in ddH₂O

Dissolve 10 g of sodium azide in 100 ml of ddH₂O. Store at room temperature for several months.

Super Broth (SB) Medium: 1% (wt/vol) MOPS, pH 7.0, 3% (wt/vol) tryptone, 2% (wt/vol) yeast extract.

Add 10 g of MOPS, 30 g of tryptone, 20 g of yeast extract in 950 ml of ddH₂O. Stir to dissolve, adjust to pH 7.0 and add ddH₂O to make the final volume 1L. Autoclave and store at 4°C for several months.

TBS: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl

Dissolve 6.05 g Tris and 8.76 g NaCl in 800 mL of H₂O. Adjust pH to 7.5 and make volume to 1L with ddH₂O. Autoclave and store at 4°C for several months.

Tetracycline Stock: 10 mg/ml in 100% ethanol.

Dissolve 100 mg of Tetracycline in 10 ml of 100% ethanol and make 1-ml aliquots.

CRITICAL: Light sensitive. Store in the dark at –20°C for several months.

Tris-HCl Solution: 1 M, pH 7.5 in ddH₂O

Dissolve 121.14 g Tris in 800 ml ddH₂O. Adjust pH to 7.0 with concentrated HCl. Bring final volume to 1 liter with ddH₂O. Autoclave and store at room temperature for several months.

Buffers for Biotinylated PTP1B Purification:

Lysis Buffer: 25 mM Na₂HPO₄, pH 6.5, 10 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 2 mM Benzamidine, 1 Complete Protease Inhibitor Tablet/50 ml, 0.5 mg/ml lysozyme

Biotin Blocking and Elution Buffer: 2 mM D-biotin in PBS

Biotin Elution Buffer: 5 mM D-biotin in PBS

Mono-Q Buffer A (Q-Buffer A): 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT

Mono-Q Buffer B (Q-Buffer B): 25 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA, 5 mM DTT

PTP1B Storage Buffer: 25 mM Tris-HCl, pH 7.0, 50 mM NaCl, 5 mM DTT, 0.1 mM PMSF (add before freezing), 0.02% (wt/vol) NaN₃, 50% (vol/vol) glycerol.

Buffers for scFv purification:

Lysis Buffer: 50 mM Na₂HPO₄, pH 8.0, 500 mM NaCl, 10 mM imidazole, 1mM PMSF, Complete EDTA free protease inhibitor cocktail.

Washing Buffer: 50 mM Na₂HPO₄, pH 8.0, 500 mM NaCl, 20 mM imidazole

Elution Buffer: 50 mM Na₂HPO₄, pH 8.0, 500 mM NaCl, 250 mM imidazole

PROCEDURE

Selection of Specific scFvs by In-Solution Panning

TIMING ~ 3 days for each panning step

- 1) Prewash streptavidin (SA) coated magnetic beads with 10 volumes of TBS containing 2% BSA and 0.02% sodium azide, by mixing and centrifuging at 12,000×g for 30 seconds. Resuspend the washed beads in TBS containing 2% BSA, 5 mM DTT and 0.02% sodium azide, to adjust the final SA concentration to 10 mg/ml.
▲ CRITICAL STEP The binding capacity of the beads is 5–10 µg (30–60 pmol) of biotinylated antibody (IgG) per mg of SA beads. Therefore, 25 µl of SA beads at 10 mg/ml, should bind 7.5–15.0 pmol of biotinylated protein.
- 2) Pre-adsorb the scFv library with SA beads in 400 µl final volume in 1.5 ml microcentrifuge tube. Mix 2×10^{12} phage particles in 400 µl of 2% BSA in TBS plus 1% Tween-20, 10 mM DTT and add 25 µl of prewashed SA beads and incubate for 2 hours at 4°C.
- 3) Add recombinant 37kDa wild type PTP1B (4 µg) in 400 µl 1× TBS, 2% BSA, 0.5% Tween-20 and 5 mM DTT to 200 µl of pre-adsorbed phage (10^{12} phage particles) and incubate for 4 hours at 4°C.
▲ CRITICAL STEP In the second panning step, use 10 µg (25-fold molar excess) of untagged wild type PTP1B (PTP1B-WT), instead of 4 µg (10-fold molar excess) used in the first round of panning. In the subsequent rounds of panning use 20 µg of untagged wild type PTP1B, which is a 50-fold molar excess of the biotinylated PTP1B-CASA.
- 4) Add the biotinylated PTP1B-CASA (400 ng) in 400 µl 1× TBS, 2% BSA, 0.5% Tween-20 and 5 mM DTT to this mixture and incubate at 4°C for 4 hours in a humidified box.
- 5) Add pre-washed SA beads (25 µl) to the phage/antigen mixture in the tube and incubate for 15 minutes at room temperature on a rocking platform.

- 6) Add 100 μ l of 1 mM biotin (~0.1 mM final) in TBS containing 2% BSA and 5 mM DTT to the solution and incubate for 5 minutes at room temperature to block remaining streptavidin binding sites on the beads.

▲ **CRITICAL STEP** This step is important in reducing the number of non-specific, bead-binding scFvs during the panning steps. Biotin at this concentration does not compete off the biotin-Streptavidin interaction and the scFv-antigen will be captured efficiently with a net reduction of non-specific scFvs.

? TROUBLESHOOTING

- 7) Aspirate the supernatant after the incubation and wash the beads 3 times by resuspending in 1 ml of TBS and centrifuging at 12,000 $\times$ g.
- 8) Elute the phage particles associated with the SA-biotinylated PTP1B-CASA complex by incubation with 100 μ l of elution buffer (0.2 M glycine-HCl pH 2.2, 1 mg/ml BSA) for 10 minutes at room temperature, with occasional pipetting up and down to mix the beads.
- 9) Hold the beads at the bottom of the tube with the magnet, collect the supernatant and transfer to a new microfuge tube and neutralize with 20 μ l of neutralization buffer (1 M Tris base, pH 9.1).
- 10) Add the eluate to infect a 2 ml overnight culture of XL-1 Blue strain of *E. coli* in SB medium and incubate at room temperature for 15 minutes.
- 11) Add 6 ml of pre-warmed (at 37°C) SB medium (+ 1.6 μ l of 100 mg/ml carbenicillin + 12 μ l of 5 mg/ml tetracycline) and transfer the culture to a 50 ml tube and shake at 37°C, 250 rpm for 1 hour.
- 12) Add 2.4 μ l of 100 mg/ml carbenicillin to the culture and shake for an additional hour at 250 rpm and 37°C.
- 13) Titer the input and output phage to estimate the number of phage particles before and after each panning step. Dilute 5 μ l of the infected culture in 500 μ l of SB medium and plate 10 μ l and 100 μ l of this dilution on LB/Amp or LB/Carbenicillin plates for output titrating. Infect 100 μ l of *E. coli* culture with 2 μ l of a 10^{-8} dilution of the phage preparation in SB. Incubate the infected culture for 15 minutes at RT and plate on an LB/Amp plate. Incubate the output and input plates at 37°C overnight.
- 14) Add the helper phage VCSM13 (10^{12} pfu) to the 8 ml culture (from step 12) and transfer to a 500 ml flask. Add 91 ml of pre-warmed (37°C) SB medium (with 46 μ l of 100 mg/ml carbenicillin and 184 μ l of 5 mg/ml tetracycline) and shake the culture at 300 rpm for 2 hours at 37°C.
- 15) Add 140 μ l of 50 mg/ml Kanamycin and continue shaking overnight at 300 rpm and 37 °C.
- 16) Centrifuge the culture at 3000 $\times$ g for 15 minutes at 4°C.
- 17) Transfer the supernatant to a clean 250 ml centrifuge bottle and add 25 ml 5 $\times$ PEG/NaCl (final 4% PEG, 0.5 M NaCl). Mix and incubate on ice for 30 minutes.
- 18) Precipitate the phage particles by centrifuging at 15,000 $\times$ g for 15 minutes at 4°C.

- 19) Drain the supernatant and resuspend the phage particles in 2 ml of 1% BSA in TBS. Filter the phage preparation using 0.20 μm disc filters.

PAUSE POINT: Phage preparations can be stored at 4°C for several weeks.

? TROUBLESHOOTING

- 20) Carry out another round of panning by repeating (steps 1–19) using the amplified phage particles. Perform a total of five rounds of panning before proceeding to step 21. Determine the input and output phage titers on LB + carbenicillin plates before and after each round of panning. Use these output titer plates (in step 21) as the source of individual phage particles expressing scFv fused to the surface protein pIII.

Sequence Analysis of Individual scFv Clones

TIMING ~5 days

- 21) Select individual clones (~100 from each panning step) randomly from the enriched scFv pools (from 2nd and subsequent round of panning) and determine the DNA sequence.
- 22) Identify different individual scFvs and sort into groups of identical sequences by multiple sequence alignment using MultAlin (<http://multalin.toulouse.inra.fr/>) software⁷⁰.

Expression and Purification of Soluble scFvs from Bacteria

TIMING ~3 days

- 23) Grow TOP10F' *E. coli* cells from single colonies representing each sorted group in step 22 in Super Broth (SB) medium at 37°C with shaking at 250 rpm overnight.
- 24) Dilute this overnight culture 1:50 in SB medium and incubate at 37°C at 250 rpm until the OD₆₀₀ becomes ~0.8.
- 25) Shake this culture slowly at 100 rpm for 15 minutes at 37°C to regenerate the sheared F' pili of the TOP10F' strain.
- 26) Infect these TOP10F' cells with phage particles expressing individual scFvs, by adding ~10¹² phage particles/ml of bacterial culture and incubate at room temperature for 15 minutes with occasional gentle shaking.

▲ **CRITICAL STEP** The use of TOP10F' cells is essential for expression and purification of scFv without pIII fusion. TOP10F' is a nonsuppressor *E. coli* strain that reads the amber codon as the stop codon and scFv is produced without being fused to pIII surface protein of phage.

? TROUBLESHOOTING

- 27) Dilute the infected culture 1:50 in SB medium with 50 $\mu\text{g/ml}$ of Carbenicillin and incubate at 37°C and 250 rpm until the OD₆₀₀ reached to 0.5.
- 28) Induce the culture with 1.5 mM IPTG at 30°C and 250 rpm for 16 hours.
- 29) Harvest the cells and resuspend in Lysis Buffer. Incubate the resuspended culture with lysozyme (0.5 mg/ml) for 30 minutes at 4°C.
- 30) Sonicate on ice for 15 sec X 3 with 1 minute interval in between (pulsing at 100% duty cycle, output control set at 5) and then for 180 seconds (pulsing at

50% duty cycle, output control set at 5). Centrifuge at 50,000×g for 30 minutes at 4°C.

- 31) Purify the soluble scFv from the supernatant using Ni-NTA agarose gravity columns. Add the supernatant to the column and wash with 20 column volumes of Wash Buffer.
- 32) Elute the bound protein with the Elution Buffer. Measure the concentration of the purified scFv by Bradford protein assay.

Reversible Oxidation of PTP1B by H₂O₂

TIMING 1 day

- 33) Mix purified PTP1B(1–321) (see Reagents) at 15 nM with increasing concentration of H₂O₂ (0.05 mM to 100 mM) in phosphatase assay buffer and incubate at room temperature for 10 minutes.
- 34) Equilibrate a Zeba Desalting Column (0.5 ml) by washing it 3 times with phosphatase assay buffer without any reducing agent. Apply 125 µl of the oxidized PTP1B to the column and collect the eluate as the sample from which H₂O₂ is removed.
- 35) Measure phosphatase activity for each protein sample treated with different H₂O₂ concentrations using ³²P-labeled RCML as the substrate, with or without 5 mM TCEP (see Box 2).

? TROUBLESHOOTING

- 36) Compare the activity of each sample to that of the untreated PTP1B in the presence of 5 mM TCEP.

Screening for Conformation-Sensor scFvs

TIMING ~2 days

- 37) Oxidize and transiently inactivate recombinant PTP1B with H₂O₂, so that the phosphatase activity can be completely restored following the removal of H₂O₂ by quick buffer exchange and addition of reducing agent (TCEP or DTT) (see Steps 33–36). After H₂O₂ treatment and removal, incubate the PTP1B with purified, bacterially expressed scFvs in 100-fold molar excess. Measure phosphatase activity under reducing conditions (see Box 2) to assess the potential of individual scFvs to stabilize the reversibly oxidized conformation.
- 38) Incubate the purified scFv (750 nM) with PTP1B (7.5 nM) under reducing conditions (5 mM TCEP) in 50 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% BSA for 30 minutes at room temperature, to assess whether the scFv binds to the reduced form of PTP1B and exerts any direct inhibitory influence on phosphatase activity.
- 39) Determine the phosphatase activity in these protein mixtures by assay with ³²P-labeled RCML as substrate (Box 2).
- 40) Incubate reversibly oxidized PTP1B (7.5 nM) similarly with individual scFvs (750 nM) for 30 minutes at RT in the same binding conditions, except in the absence of reducing agent, to determine the effect of scFv binding on the reduction and reactivation of PTP1B-OX.
- 41) Determine PTP1B activity in each sample using 100 nM ³²P-RCML as the substrate, with or without 5 mM TCEP reducing agent (Box 2).

Ni-NTA Pull-down Experiments to Determine the Binding of scFvs to PTP1B-OX

TIMING 3 days

- 42) Oxidize purified PTP1B (50 nM) with 250 μ M H₂O₂, followed by a quick buffer exchange to remove H₂O₂ (see steps 33–36).
- 43) Incubate purified scFv in 100-fold molar excess over PTP1B-OX or PTP1B-CASA or with PTP1B under reducing condition (with 2 mM TCEP) in binding buffer for 2 hours at 4°C.
- 44) Add Ni-NTA agarose (50 μ l as 50% slurry equilibrated in the binding buffer) and incubate for one hour at 4°C. Alternatively, perform similar experiment using anti-HA High Affinity antibody (clone 3F10) coupled to agarose beads (50 μ l as 50% slurry) to capture scFv-PTP1B-OX complex from solution.
- 45) Pull down protein complex bound to Ni-NTA/anti-HA agarose beads and wash (three times, 5 minutes each, at 4°C) with binding buffer containing 20 mM imidazole.
- 46) Elute the protein complex from the Ni-NTA agarose beads with 500 mM imidazole (in binding buffer) for 15 minutes at 4°C with gentle shaking. For anti-HA agarose beads add 50 μ l of 2 $\times$ sample loading buffer and boil the sample for 5 minutes at 100 °C.
- 47) Separate the complex by SDS-PAGE and detect PTP1B with anti-PTP1B antibody (FG6) and scFv with anti-HA antibody [anti-HA (3F10)-HRP] by immunoblotting.

? TROUBLESHOOTING

TIMING

Box 1, PTP1B biotinylation in vivo (cloning of the biotinylation tag, expression and purification): 2 weeks

Steps 1–19, in-solution panning: 3–4 days for one step of panning

Box 2, Phosphatase assay: substrate preparation and assay: 2 days

Step 21–22, Sequence analysis of individual scFv clones: ~5 days

Steps 23–32, Purification of scFvs from E. coli: ~3 days

Steps 33–36, Reversible oxidation of PTP1B by H₂O₂: 1 day

Steps 37–41: Screening for conformation-sensor scFvs in vitro: 2 days for each round of screening

Steps 42–47: Ni-NTA and HA-agarose pull-down experiments: 3 days

TROUBLESHOOTING

Troubleshooting advice is provided in Table 1.

ANTICIPATED RESULTS

Size of the library

The size of the scFv library is generally defined by the number of total transformants. The expected library size for successful panning and isolation of candidate scFvs is 10^6 . The final library size in our case was $\sim 2 \times 10^7$. If the size of the scFv library is below 10^6 , new transformation should be performed using freshly prepared electrocompetent cells with optimized protocol. It is also possible to combine multiple ligations and transformations of the scFv-phagemid construct to increase the library size.

Sequence analysis of the scFvs, diversity of the library, post-panning sequence enrichment

After each round of panning the phage titer should be carefully monitored. Approximately 10^{13} phage should be obtained from 100 ml of supernatant after each panning step. XL1-Blue or TOP10F' E. coli can be infected by diluted phage preparations and plated on LB agar plates with carbenicillin to measure the phage titer. Alternatively, phage titers can be estimated by measuring absorption at 269 nm⁶². It is critical to maintain the number of phage particles at least $10^2 - 10^4$ times greater than the library size (e.g., at least 10^9 cfu for a library of 10^7 clones). This ensures that the original diversity is preserved before the panning and enhances selection of scFv clones that would bind to different epitopes of the target antigen. Increased phage output/input ratio after each round of panning generally indicates enrichment of specific scFvs. Using this protocol, approximately 1000-fold enrichment of the scFvs after the 4th round of panning was observed (Figure 3b). With an input of $10^{11} - 10^{12}$ phage, the expected output titer after the first and second round of selection is generally in the range of $10^5 - 10^7$ phage. After the 3rd and 4th round of panning this number is expected to increase to $10^7 - 10^9$. If the phage titer falls out of this range the selection procedure should be optimized. Generally, if an excessive number of panning steps are performed (more than five rounds), dominant and potentially unwanted clones may emerge.

The average scFv insert length is approximately 700 bp, encoding the conserved framework region and variable CDRs. We sequenced ~400 randomly selected scFv clones after different panning steps using phagemid-specific primers. In our case, 95% of the sequences were full-length, functional scFvs, indicating that the cloning strategy and library display worked appropriately. In cases where scFvs are found with frequent incomplete sequences, immature stop codons before the C-terminal 6His or HA tags or no scFv inserts at all, the library should be reconstructed. Sequences should be sorted into groups on the basis of differences in their hypervariable regions. Selection of functional scFv sequences can be confirmed by checking that the sequences are from the animal that was immunized to generate the library. All the selected sequences in our case aligned with the amino acid sequence of light and heavy chains of chicken IgG, except for the differences in the hypervariable regions. The most variable segment in the scFvs is expected to be in the CDR3 region of the heavy chain. The selected scFv sequences should also contain the 6-His and HA tags at the C-terminus, the presence of which in frame with the scFv sequence is an indication that the library cloning and ligation worked. It is advisable to check for clonal diversity after the 2nd and subsequent rounds of panning. Using our protocol, diversity among the functional scFv sequences was found to be ~70% after the 2nd round and ~20% after the 4th round of panning (Figure 3c).

Selection of candidate scFvs from *in vitro* screening (Figure 4)

Using this protocol, positive clones are expected after as few as two rounds of selection. This outcome may vary depending on the antigen quality and property, binding conditions,

blocking strategy, washing stringency and elution conditions. For rapid screening, we expressed individual scFvs from enriched library in *E. coli* using the phagemid vector (pComb3XSS) that was used for constructing the library. Purification of soluble scFv from a small-scale culture (10 ml) is expected to yield 0.01–0.1 mg of purified scFv with 50–75% purity as assessed by SDS-PAGE. If desirable purity of the scFv is not achieved from the bacterial lysates, it can be isolated from periplasmic fractions.

When incubated with reversibly oxidized PTP1B, the candidate scFvs are expected to demonstrate substantial inhibition (50%) of reactivation (Figure 4c). None of the selected scFvs should have direct inhibitory effect on the phosphatase activity of PTP1B under reducing conditions. Initially, we have performed the screening for specific scFvs from the fourth and fifth round of panning; however, candidates could be isolated as early as from the second round of panning. In fact, some of the clones that were found after the 4th round of panning were also identified after the second round. This indicates that the enrichment and selection criteria in this protocol were sufficient for isolation of specific binders from as early as second round of panning.

The scFvs that are selected by the primary screening are expected to bind the oxidized protein but not the reduced active enzyme. As a control, binding with PTP1B-CASA was also tested and scFvs specific to PTP1B-OX should bind to the mutant protein, which is structurally identical with the oxidized enzyme (Figure 4d). In similar binding experiments scFvs that did not stabilize the inactive conformation in the enzymatic screening assay, should not demonstrate binding to the oxidized enzyme. In addition, one can also determine the binding constants of individual scFvs by using interaction analysis techniques such as Surface Plasmon Resonance (SPR). Typically, scFvs isolated from an immunized library against human antigens have affinities in the nanomolar range.

Specificity of the scFvs

The closest relative of PTP1B in the PTP family is TCPTP, which displays 75% sequence identity within the catalytic domain³². This approach generated scFvs with remarkable selectivity that could distinguish between these two closely related enzymes. Currently there is no crystal structure of oxidized TCPTP; however, overlap of the crystal structure of the active, reduced form of these two closely related PTPs illustrated some structural differences. Conformational changes associated with oxidation are likely also to reveal additional structural attributes that contribute to this specificity. Use of the entire catalytic domain of PTP1B-OX as the antigen to construct the scFv library and for subsequent screening was likely important. We do not anticipate that this selectivity would have been achieved if the scFvs were generated and screened for using the oxidized peptide of the active site catalytic motif, which is well conserved in PTP family.

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Box 1: Biotinylation of PTP1B

TIMING ~ 2 weeks including cloning, expression and purification

We expressed PTP1B with an N-terminal biotinylation sequence from a pET19b vector in *E. coli* [BL21(DE3)] together with the pBirAcm plasmid that harbors recombinant biotin ligase.

1. Using standard cloning strategies, PCR amplify the catalytic domain (residues 1–321) of the WT or CASA mutant with the biotinylation tag with an additional Nco I restriction site at the 5' of the tag and clone in pET19b plasmid to make the expression construct for NBTPTP1B (N-terminally biotinylated-PTP1B). The primers used for this amplification are–

5'GGGGAACCATGGGCCTGAACGACATCTTCG

AGGCTCAGAAAATCGAATGGCACGAAATGGAGATGGAAAAGGA
GTTTCG3' (sense) and

5

'AGCAGCCGGATCCCCGGGCTGCAGGAATTCTCTAGACTAGAG3'
' (antisense)

2. Using standard methods, transform competent BL21(DE3) with the recombinant biotin ligase (BirA)-containing plasmid pBirAcm and maintain under the selection of chloramphenicol (10 µg/ml) on LB agar plate.
3. Make pBirAcm-containing bacteria (from step 2) chemically competent again using standard method and transform with the NBT-PTP1B constructs from step 1. Maintain the doubly transformed cells under the selection of both chloramphenicol (10 µg/ml) and ampicillin (100 µg/ml) on LB agar plate.
4. Induce expression of recombinant proteins with 1 mM IPTG in presence of 50 µM biotin in the growth medium for 4 hours at 37°C.
5. In the first step of purification, separate the biotinylated recombinant protein by immobilized monomeric Avidin and elute with 5 mM biotin in PBS following manufacturer's instruction.
6. In the second step, further purify the protein by HiTrap Q FF anion exchange chromatography using standard FPLC protocol and following manufacturer's instruction. Elute the protein in Q buffer B. Dialyze the protein in PTP1B storage buffer overnight at 4°C.
7. To ensure efficient recovery of the antigen under the panning conditions and to assess the efficiency of the biotinylation *in vivo*, check NBT-CASA or NBT-WT in a Streptavidin pull down experiment.
8. Mix the biotinylated proteins (10 nM final) with 25 µl of Streptavidin coated magnetic bead in 1 ml of TBS containing 2% BSA, 5 mM DTT, 0.5% Tween-20 and 0.02% sodium azide and incubate with gentle rotation at 4°C for 2 hours.
9. Wash the beads 3 times with cold TBS (2% BSA, 5 mM DTT, 0.5% Tween-20 and 0.02% sodium azide) and separate the proteins by SDS-PAGE. Detect PTP1B by blotting with anti-PTP1B antibody (FG6) and the biotinylated protein with anti-biotin-HRP antibody.

Box 2: Phosphatase Assay to Determine the Activity of PTP1B

TIMING ~2 days including substrate preparation and enzymatic assay

Reduced Carboxamidomethylated and Maleylated Lysozyme (RCML) was used as a substrate in a phosphatase assay^{53, 54} for activity-based screening for PTP1B-OX conformation sensor scFvs. Other substrates of PTP1B could also be used in the assay.

1. Phosphorylate Reduced Carboxamidomethylated and Maleylated Lysozyme (RCML) with ^{32}P using recombinant GST-FER kinase and $[\gamma\text{-}^{32}\text{P}]$ ATP to stoichiometry of ~0.8 mol ^{32}P incorporated/mol of protein, as described in⁵⁵.
2. Mix PTP1B (1–321)-WT without any modification and PTP1B (1–321)-WT or CASA with Nterminal biotinylation *in vivo* in *E. coli* (5 nM PTP1B final concentration) with 500 nM ^{32}P RCML in phosphatase assay buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% BSA, 5 mM TCEP) and incubate at 30°C for 10 minutes.
3. Stop the reaction with 10% (vol/vol) TCA (final) with 2.5% (wt/vol) BSA, incubate at –80°C for 30 min.
4. Thaw the reaction at room temperature, spin at top speed in a microcentrifuge for 5 minutes and collect an aliquot of the supernatant.
5. Determine the release of ^{32}P -phosphate by scintillation counting. Compare the specific activity of the protein preparations with that of the untagged PTP1B (1–321), activity of which was set as 100%.

? TROUBLESHOOTING

Figure 1. Generation of Single Chain Variable Fragments (scFvs) for PTP1B-OX

(a) Oxidation-induced conformational change in PTP1B. The panel illustrates a close-up surface representation of the catalytic core of the reversibly oxidized, inactive state of PTP1B, highlighting the conformational re-arrangements in yellow (adapted from⁷¹). Oxidation-induced formation of the sulphenyl-amide intermediate triggers dramatic structural changes in the catalytic site of the protein^{39, 40}. Tyr46 of the pTyr loop, which defines the depth of the catalytic cleft, and the PTP loop containing the signature catalytic motif, which is located at the base of the cleft under reducing conditions, both adopt a solvent exposed position (shown in yellow) in the oxidized state. In PTP-CASA, mutation of both the catalytic cysteine 215 and serine 216 to alanine breaks the same hydrogen bonds that are disrupted by oxidation of the active site cysteine, which induces a conformation that is identical to that of the oxidized enzyme⁴⁵. **(b)** A flexible peptide linker joins the variable light (V_L) and variable heavy (V_H) regions of antibody to generate the scFv [adapted from⁴⁵].

Figure 2. Site-Specific Biotinylation of PTP1B (Wild Type and CASA mutant)

(a) Site-specific, *in vivo* biotinylation did not affect PTP1B activity. Phosphatase activity of recombinant PTP1B (1–321), which was biotinylated *in vivo* (NBT-1B-WT or NBT-1B-CASA) or *in vitro* (1B-WT+BT) was measured using ^{32}P -RCML as the substrate. The activity of the different PTP1B preparations was compared to that of the untagged wild type enzyme (1B-WT), which was set as 100%. Error bars represent standard deviation from 4 separate phosphatase assays. (b) The biotinylation sequence is a 15-residue unique peptide, which is recognized by biotin ligase. The biotinylation tag was fused to the N-terminal of PTP1B in pET19b vector and co-expressed in *E. coli* with the recombinant biotin ligase (BirA) by IPTG induction. In the presence of ATP, the ligase specifically attaches biotin to

the lysine residue in this tag, which enables homogenous, site-specific *in vivo* biotinylation⁴⁸. Addition of biotin during the time of induction ensures biotinylation of the tagged protein. **(c–e)** Purification of biotinylated PTP1B (1–321, WT and CASA). **(c)** In the first step, the biotinylated recombinant protein was enriched on monomeric avidin beads and eluted (with 5 mM biotin) fractions were collected. For PTP1B-WT, the corresponding protein fractions demonstrated phosphatase activity as measured by dephosphorylation of the low Mr substrate pNPP, whereas, as expected, the fractions for the CASA mutant showed no activity. In the second step **(d)** the protein was further purified on a Q anion exchange column and eluted with an NaCl gradient. **(e)** Purified biotinylated proteins were analyzed by SDS-PAGE and Coomassie Blue staining. The arrow indicates the migration of PTP1B protein. **(f)** The efficiency of biotinylation, and the recovery of the biotinylated protein from solution by Streptavidin coated magnetic beads, were tested under experimental conditions similar to those used in the in-solution panning steps for enrichment from the antibody phage display library. NBT-1B-CASA (10 nM) was captured with Streptavidin coated magnetic beads and presence of biotinylated protein (and PTP1B) was detected in input (Inp), supernatant (Sup) and precipitated (Ppt) sample by immunoblot using anti-biotin-HRP and anti-PTP1B antibodies. In the last three lanes (Inp, Sup and Ppt of NBT-1B-CASA) 4× more samples were used to increase the load for better detection of the biotinylated proteins.

Figure 3. In-solution Panning to Enrich PTP1B-OX-Specific scFvs

(a) Schematic representation of the panning steps. Single chain variable fragment (scFv) sequences were inserted into the phagemid that also encodes a recombinant phage coat protein gene III (at the C-terminus of the scFv), and transformed to a male strain of *E. coli* (XL1-Blue) harboring F' pili. Infection of these transformed bacteria with helper phage (VCSM13) enabled them to make a collection of functional phage particles expressing the scFvs fused to the surface protein pIII. A phage particle displaying a functional antibody fragment in the form of scFv as a fusion protein with the coat protein pIII is shown in the inset [adapted from⁴⁵]. In the in-solution panning, up to 50-fold molar excess of wild type

PTP1B over the CASA mutant was added under reducing condition. Then, biotinylated PTP1B-CASA was added and specific phage scFvs were isolated by magnetic separation. The phage-scFvs were eluted by glycine-HCl, pH 2.2, amplified in bacteria and used for subsequent rounds of panning to enrich the library pool with CASA-specific scFvs. **(b)** Phage output/input ratio was estimated by counting plaque forming units representing the phage particles before and after each round of panning to assess scFv enrichment **(c)** As the library was enriched, the diversity decreased. Diversity was determined by checking the variation in the CDR regions of sequenced scFvs from different round of panning. **(d)** A flow chart summarizes the screening, isolation and characterization of PTP1B-OX-specific scFvs.

Figure 4. Screening of scFvs Specific to PTP1B-OX

(a) Conditions were established for reversible oxidation of PTP1B by H₂O₂ in vitro. In this assay PTP1B (15 nM) was incubated with H₂O₂ as indicated to oxidize the enzyme. Following buffer exchange to remove H₂O₂, the inactivation and reactivation of the oxidized enzyme samples (5 nM final) was determined by the phosphatase assay without or with TCEP, respectively. (b) Flow chart shows the steps for screening conformation sensor scFvs against reversibly oxidized PTP1B in vitro. (c) Reversibly oxidized PTP1B samples (7.5 nM) were incubated with individual scFvs (750 nM) at room temperature for 30 minutes. Reactivation of enzyme was measured by phosphatase activity assay in presence of

reducing agent (TCEP). The final enzyme and scFv concentrations in the phosphatase assay were adjusted to 5 nM and 500 nM, respectively. In **(a)** and **(c)** error bars show standard deviation from three phosphatase assays. **(d)** Specific binding of scFv45 to oxidized PTP1B *in vitro*. Reversibly oxidized (1B-OX), reduced (1B-R), and mutant form (1B-CASA) of PTP1B were incubated with purified scFv 45 and the protein complex was captured using Ni-NTA agarose beads. The input, supernatant and eluate were separated by SDS-PAGE and PTP1B and scFv in the complex was detected by immunoblotting with anti-PTP1B or anti-HA-HRP antibodies, respectively.

Table 1

Troubleshooting

Steps	Problem	Possible Reason	Solution
Box 1	Insufficient biotinylation of NBT-PTP1B expressed in <i>E. coli</i>	<p>Biotin ligase is not adequately co-expressed</p> <p>Either of the expression plasmids is lost</p> <p>Improper expression conditions for either or both of the proteins (biotin ligase and PTP1B)</p>	<p>Use different but compatible origins of replication and independent selection markers for maintenance of the plasmids expressing biotin ligase and the target protein.</p> <p>Make sure that the expression strain is maintained under two independent antibiotic markers (ampicillin for pET19b-PTP1B and chloramphenicol for pBirAcm)</p> <p>Optimize the expression conditions systematically by using appropriate expression strain and adjusting the IPTG concentration and the duration and temperature of induction to improve the yield and solubility of the co-expressed proteins.</p>
Step 6	Low enrichment of specific scFvs/ high enrichment of non-specific scFvs	Non-specific binding of the phage to the beads, inadequate wash conditions	After the antigen-scFv-phage complex is captured by the Streptavidin magnetic beads, add biotin (0.1 mM) to block the remaining Streptavidin sites to compete off phage that bind to the beads non-specifically. Increase the Tween-20 (up to 0.5%) in the wash buffer.
Step 13	Very few colonies after the panning steps	<p>Low infectivity of the library phage preparation</p> <p>Reduced infectivity of the helper phage preparation</p> <p>Loss of infectivity of the library phage during acid elution</p> <p>Loss of F' pili of the bacterial culture over time</p>	<p>Use freshly prepared phage for panning. Stored phage should be reamplified before panning.</p> <p>Check the titer of the helper phage by plaque forming assay. Use helper phage in the final concentration of 10^{12} pfu/ml.</p> <p>Do not allow the incubation for elution with Glycine-HCl to go past 10 minutes as phage infectivity may decrease rapidly after that time. Neutralize the eluted phage immediately.</p> <p>Allow culture of bacteria for an additional 15 minutes of slow shaking at 37°C and 100 rpm for regenerating sheared F' pili before infection with the phage.</p>
Step 19	<p>Difficulties in filtering the phage preparation; filter clogs frequently</p> <p>Low yield of the scFv-phage</p>	<p>Contamination in the phage preps</p> <p>Too concentrated phage. Phage in solution tend to precipitate out if the preparation is $>10^{13}$ pfu/ml</p> <p>Contamination by environmental bacteriophage</p>	<p>Add 0.02% sodium azide (w/v) in the stored phage preps</p> <p>Spin the phage solution after resuspending the precipitated pellet. Use 0.45 μM filter instead of 0.20 μM. Maintain the phage preps in a concentration $\sim 10^{12}$ pfu/ml</p> <p>Use aerosol-resistant pipet tips for all phage protocols and wear gloves for every step. Wipe the work area with ethanol regularly.</p>
Box 2	<p>High background in RCML phosphatase assay</p> <p>Low phosphatase activity</p>	<p>The ^{32}P-RCML prep has high amount of unincorporated 32p</p> <p>PTP1B prep has low activity because of improper storage.</p>	<p>Remove unincorporated ^{32}P from the RCML substrate either by desalting with Sephadex G-25 column (PD-10 columns from GE) or by TCA precipitation and resolubilization.</p> <p>Store purified PTP1B in the proper storage buffer at -80°C and avoid repeated freeze-thaw cycle. Add BSA (0.05%) in the phosphatase assay buffer to prevent PTP1B adhering to the reaction tube.</p>
Step 32	Low yield of scFvs from bacteria	<p>Proper bacterial strain for expression is not used.</p> <p>The expressed scFvs may have gone into inclusion bodies.</p>	<p>Use a nonsuppressor bacterial strain so that the amber codon is read as a stop codon and scFv is expressed without pIII fusion.</p> <p>Optimize the conditions to express soluble scFv by adjusting IPTG concentration, induction temperature and time.</p>

Steps	Problem	Possible Reason	Solution
<i>Step 36</i>	Incomplete reactivation of PTP1B-OX after adding the reducing agent	Inadequate removal of H ₂ O ₂ after oxidation. The reducing agent is inactive.	Equilibrate the desalting column properly and desalt according to the recommended protocol for the column. Use freshly prepared DTT or use the frozen stock without multiple freeze thaw cycles. Alternatively, use TCEP as the reducing agent as it is stable at room temperature.