Fluorescence-Based Cloning of a Protein Tyrosine Kinase with a Yeast Tribrid System

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Post-translational modifications of proteins control myriad biological functions. However, relatively few methods exist for the identification of the enzymes that catalyze these modifications. To expand this repertoire, we report a yeast genetic approach that enables the identification of protein tyrosine kinases (PTKs) from cDNA libraries. Yeasts were transformed with four vectors encoding: 1) a potentially universal PTK substrate fused to the LexA DNA binding domain, 2) the Grb2-SH2 domain fused to the B42 activation domain, 3) a fluorescent reporter gene controlled by LexA DNA sites, and 4) a Jurkat cDNA library. Transient expression of PTKs, such as the lymphocyte-specific kinase Fyn, resulted in phosphorylation of the DNA-bound substrate, recruitment of the Grb2-SH2 domain, and activation of the fluorescent reporter gene. This brief induction of protein expression circumvented the potential toxicity of PTKs to the yeast. Fluorescence activated cell sorting (FACS) enabled isolation of PTKs, and these enzymes were further characterized by flow cytometry and immunoblotting. This approach provides a potentially general method for the identification and evaluation of enzymes involved in the post-translational modification of proteins.

Introduction

Covalent post-translational modifications of specific amino acid residues control numerous biological functions including cellular proliferation, programmed cell death, and cellular responses to environmental stimuli.[1–5] These modifications often create recognition elements that bind other biomolecules and control patterns of enzymatic activity in cells. For example, the phosphorylation of specific protein tyrosine residues by protein tyrosine kinases (PTKs) creates docking sites for cognate protein modules, such as SH2 domains. These domains bind tightly to phosphotyrosine residues within specific peptide sequences to propagate numerous signaling events. Despite the critical importance of post-translational modifications of proteins in cellular biology, proteomic efforts to identify the enzymes that catalyze these modifications and define the protein interactions that are dependent on these modifications remain largely unexplored.

Yeast genetic systems such as the two-hybrid system (Figure 1 A) are powerful tools for protein interaction studies. The power of this approach derives from the ability of yeast cells to be readily transformed en masse with genetically encoded libraries that can in turn be rapidly screened to identify proteins that interact with a specific target. This is generally accomplished by expressing each member of the library as a fusion protein with an activation domain (AD). This AD component is capable of interacting with the cellular transcriptional machinery and activating gene expression if recruited to specific enhancer DNA sites in the nucleus. The target protein is generally expressed in yeast, fused to a DNA-binding domain (DBD). This DBD element binds DNA sites upstream of a reporter gene, such as *lacZ*, which encodes β-galactosidase, and enables colorimetric and other assays of the protein interactions. Strong noncovalent interactions between the DNA-bound protein and a member of the library that is fused to the AD, can reconstitute a functional transcription factor (TF). This TF recruits the cellular transcriptional machinery and activates the expression of the reporter gene. Because each cell, in theory, expresses a unique member of the library, large numbers of yeast cells can be rapidly screened to identify putative proteins that interact with the target protein.

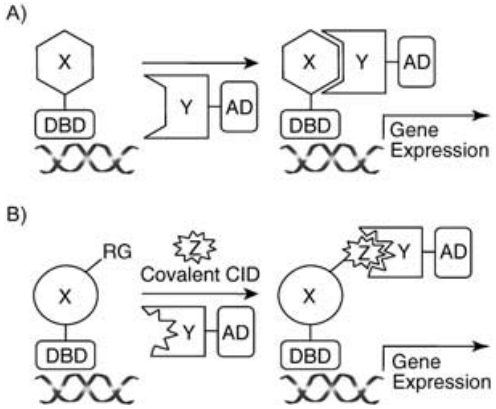

Figure 1. Representations of typical yeast-genetic systems in which dimerization of fusion proteins X and Y activates reporter gene expression. A) Yeast two-hybrid system, B) yeast tribrid system—an adaptation incorporating an enzyme or a reactive small molecule (Z) that covalently modifies protein X to create a docking site for protein Y. DBD: DNA binding domain, AD: transcriptional activation domain, CID: chemical inducer of dimerization, RG: reactive group.

The yeast two-hybrid system has been extensively employed to define protein interaction networks of model organisms.[6,7] However, this approach is typically unable to detect protein

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interactions that are dependent on post-translational modifications.[8] To enable studies of post-translational modifications in yeast, the tribrid system was developed (Figure 1 B).[9,10] In these systems, three functional components are expressed to activate reporter gene expression. These components typically include: 1) a DBD fused to a target substrate protein that bears the amino acid(s) involved in a post-translational modification event, 2) an AD fused to an adapter protein that binds the modified amino acid, and 3) an enzyme that catalyzes the post-translational modification. These enzymes typically employ cofactors, such as ATP, that covalently modify a specific amino acid. Modification of this amino acid creates a docking site for the adapter protein, resulting in the recruitment of the activation domain and expression of the reporter gene.

Yeast tribrid systems and other related genetic methods have been employed to identify adapter proteins involved in signal-transduction cascades[9,10] and chromatin-remodeling processes.[8] In signaling research, these systems have primarily been used to identify SH2 domains that bind phosphotyrosine. This post-translational modification is catalyzed by PTKs that transfer the γ-phosphoryl group of ATP to specific tyrosine residues of substrate proteins.

We sought to adapt the yeast tribrid system to isolate PTKs from cDNA libraries. The identification of these enzymes is of interest because they play key roles in signal transduction[11,12] and comprise a major class of therapeutic drug targets.[13,14] Previously reported methods for the identification and cloning of PTKs from cDNA libraries have primarily employed PCR and Southern blotting to exploit the sequence homology of identified genes.[15–17] In addition, functional methods have been reported that detect autophosphorylation of PTKs with immunoreagents.[18] More recently, mining of computer databases with programs such as BLAST[19] have been used to identify these genes and have provided estimates that the human genome encodes approximately 100 PTKs.[12,20]

Our objective was to identify yeast cells that express PTKs by screening cDNA libraries using fluorescence activated cell sorting (FACS). Precedent for this approach includes other screening strategies with yeast that were transformed with reporter genes that encode green fluorescent protein (GFP).[21] However, these systems typically detected reporter-gene expression by exposure of yeast colonies that grow on solid media to ultraviolet light. This approach requires an extended period of growth under conditions that induce protein expression, thus restricting the analysis to proteins that are not toxic to yeast and that allow colony formation.

To enable the identification of yeast cells that express potentially toxic PTKs, we modified a previously reported yeast tribrid system.[22,23] This system permits transient expression of PTK activity, detection of this activity with a potentially universal alanine-rich PTK substrate, and incorporates a fluorescent reporter gene for the isolation of cells that express PTKs by using FACS. By screening a Jurkat cDNA library against this system, the lymphocyte-specific PTK Fyn was identified, thus demonstrating the utility of this approach for the identification of enzymes that catalyze specific post-translational modifications.

Results

Design and construction of a yeast tribrid system for fluorescence-based cloning of PTKs

Expression of PTKs in yeast is generally toxic.[24] As a consequence, yeast expressing these enzymes do not typically form colonies on solid media. Hence, the isolation of yeast that express these proteins requires a nontraditional screening approach. To provide an alternative strategy, we investigated the utility of transient protein expression, protein expression analysis by flow cytometry,[25–27] and isolation of individual yeast cells by FACS. For these experiments, we used yEGFP (yeast enhanced green fluorescent protein) as reporter of gene expression. This GFP derivative is codon-optimized for expression in yeast and is suitable for the isolation of individual cells by FACS.[28]

To enable screening of yeast by FACS, we employed the control regions from the commercially available reporter vector pSH18-34. This vector includes a 2-micron origin of replication, a *URA3* selection marker, and four *colE1* operators (containing eight LexA binding sites) upstream of the *GAL1-GAL10* promoter which drives the expression of *lacZ*.[29–31] PCR was used to amplify the four *colE1* operators and the *GAL1-GAL10* promoter region of pSH18-34. This cassette was inserted upstream of the *yEGFP* gene in plasmid pBC103[32] to afford the leucine-selectable reporter vector, pDCLryEGFP.

The detection of PTKs with a fluorescent reporter vector required a low level of intrinsic cellular fluorescence. However, our previously reported yeast tribrid system[22] employed GFP as a spacer element for the display of PTK substrates to cognate enzymes. This previously reported system expressed a PTK substrate comprising LexA fused to GFP in turn linked to a tetrameric repeat of the tyrosine-containing substrate peptide sequence, AAYANAA. As an approach to eliminate this fluorescent spacer protein, an analogous LexA-$(AAYANAA)_4$ substrate that lacked the GFP spacer element was investigated. However, GFP was found to be essential for the efficient display of the $(AAYANAA)_4$ peptide substrate to PTKs expressed in yeast (data not shown). This observation was previously reported in other studies of GFP-linked PTK substrates.[33] As an alternative approach, we mutated Tyr66 of GFP to phenylalanine. This mutation is known to decrease the fluorescence of this protein without affecting its overall structure.[34,35] v-Abl kinase phosphorylated this nonfluorescent protein substrate (LexA-NFP-$(AAYANAA)_4$) and our previously reported fluorescent substrate (LexA-GFP-$(AAYANAA)_4$) when expressed in yeast (data not shown).

The yeast tribrid system shown in Figure 2 was employed to analyze and isolate PTKs by using flow cytometry and FACS. Phosphorylation of the DNA-bound $(AAYANAA)_4$ substrate was designed to trigger binding by the B42-AD–Grb2-SH2 fusion protein. The resulting complex, bound to LexA DNA sites of the reporter gene, was positioned to activate *yEGFP* expression, and render yeast cells fluorescent green. This B42–SH2 component was expressed from a novel yeast plasmid (pDCUAD) to provide compatibility with commercially available

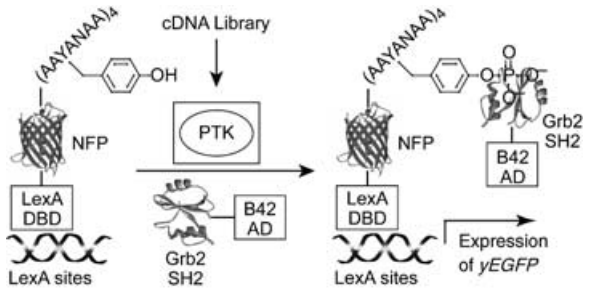

Figure 2. The yeast tribrid system incorporating a universal protein tyrosine kinase (PTK) substrate. This assay reports PTK activity by expression of the fluorescent yEGFP protein. NFP: nonfluorescent protein, $(AAYANAA)_4$: tetrameric PTK substrate.

cDNA libraries and to supply a method for eliminating false positives. The uracil selection marker of pDUCAD was chosen to enable subsequent removal of this plasmid by counterselection with growth media that contained 5-fluoroorotic acid (5-FOA).[36] In addition, as positive controls, the PTKs v-Abl and v-Src were expressed from the commercially available yeast plasmid pJG4-5.[37] This vector is compatible with LexA-based yeast two-hybrid screening systems that harbor diverse cDNA libraries.

Analysis of yeast tribrid systems by flow cytometry and FACS

Traditional screens of cDNA libraries with yeast two-hybrid systems involve multiple steps. These steps include transformation of the host strain with cDNA library and other plasmids, incubation of transformants for several days to generate colonies, replica plating of colonies to eliminate false positives, and analysis of gene products to confirm the identity and function of protein hits. In an effort to simplify some of these steps, we investigated fluorescence-based screening by using FACS with yeast transformed with a fluorescent reporter gene. This flow cytometry-based approach was chosen because of its potential to rapidly quantify the cellular fluorescence of individual yeast cells.[25–27] Moreover, the ability to isolate single fluorescent cells from a heterogeneous culture of yeast could potentially streamline the identification of hits. The method that we employed, outlined in Figure 3, comprised the following steps: 1) transformation of the cDNA library into yeast that harbor the tribrid-system plasmids; 2) incubation in liquid selection media for two days to expand the population of transformed cells; 3) a brief 4 h induction of protein expression to circumvent the toxicity associated with PTK expression, but also to activate the fluorescent reporter gene in cells that express these enzymes; 4) isolation of putative hits by FACS onto solid yeast media; and 5) further analysis of hits by replica plating onto solid counter-selection media that contained 5-FOA. This step removed the plasmid encoding the B42–SH2 fusion protein, which is required for activation of the reporter gene by a PTK, and provided a method to reduce false positives.

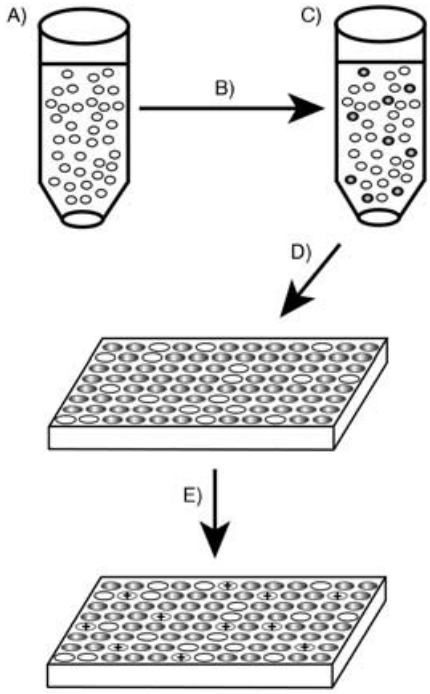

Figure 3. Protocol for screening cDNA libraries against a yeast tribrid system to identify PTKs. A) Yeast harboring tribrid-system plasmids are transformed with the cDNA library. B) The cells are incubated in selection media for approximately 2 days. C) Protein expression is induced for only 4 h so as to activate *yEGFP* expression but minimize the effect of toxic cDNA members. D) Fluorescent yeast are deposited onto solid noninducing selection media by FACS. Colonies appear after approximately 3 days. Gray wells reflect green fluorescence after growth in liquid culture under protein-inducing conditions. E) Yeast are replica plated onto solid media that contains 5-FOA; this removes the gene encoding B42–SH2. The requirement of this gene for cellular fluorescence, as analyzed by flow cytometry after growth in liquid culture, is represented by +.

The dynamic range of the fluorescent reporter plasmid pDCLryEGFP was quantified to assess its utility for cDNA library screening. As a positive control, B42 AD was expressed fused to LexA DBD in yeast that were transformed with this reporter, and cellular fluorescence was analyzed by flow cytometry. As shown in Figure 4A, 32% of the yeasts that expressed B42–LexA were in the fourth (top) decade of fluorescence. Similarly fluorescent yeasts were not observed when B42 was expressed alone. Correspondingly, when the positive control PTKs, v-Abl and v-Src, were expressed in yeast tribrid systems that contained this fluorescent reporter (Figure 4B and C), 12–16% of the cells were detected in the fourth decade of fluorescence. This fluorescence provided a threshold for isolation of individual cells by FACS. These results validated the use of pDCLryEGFP as a reporter vector that is suitable for flow cytometry and FACS screening experiments.

Identification of Fyn kinase from a human T-cell cDNA library screened against a yeast tribrid system

The yeast tribrid system shown in Figure 2, equipped with the potentially universal PTK substrate and a fluorescent reporter

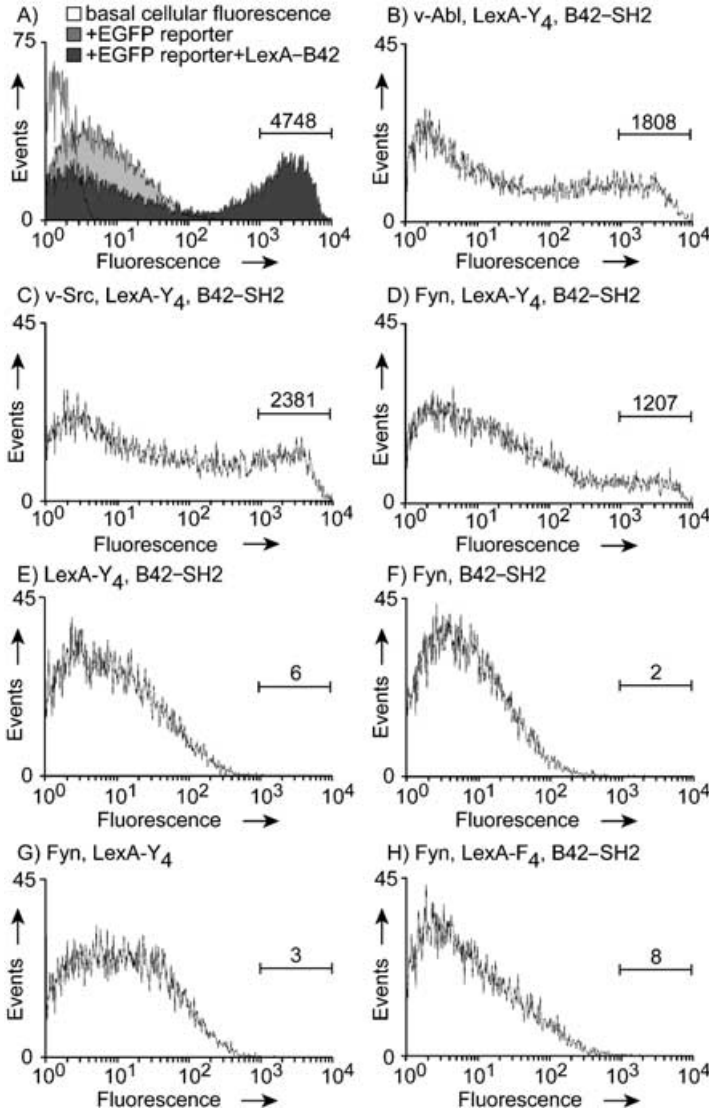

Figure 4. Flow cytometry analysis of yeast transformed with a fluorescent reporter gene. The gate shown, quantified the number of cells out of 15 000 in the fourth (top) decade of fluorescence. A) Validation of the fluorescent reporter gene. The basal fluorescence of yeast that lack the reporter gene (white), background fluorescence contributed by the yEGFP reporter alone (light gray), and the maximal fluorescence activated by a B42–LexA fusion protein (dark gray). B–D) Expression of yEGFP in yeast tribrid systems. B) Positive control v-Abl (237–630) C) positive control v-Src (137–526); and D) Fyn kinase. E–H) Fyn kinase omission control experiments. LexA-Y_4 represents the tetrameric tyrosine-containing universal substrate. LexA-F_4 represents a tetrameric phenylalanine-containing control sequence.

gene, was investigated as a tool for cloning PTKs from a human Jurkat cDNA library. Transformation of yeast following the protocol outlined in Figure 3, yielded fluorescent yeast that were sorted by FACS onto solid noninducing yeast-selection media in a 96-cell per plate format. Sorting onto two of these plates yielded 159 viable colonies. These colonies were replica plated onto solid yeast counterselection media that contained 5-FOA to remove the B42–SH2 expression vector. Comparative analysis by flow cytometry of yeast colonies on master plates with those on 5-FOA plates identified a subset of colonies that appeared to require the B42–SH2 protein for the expression of the reporter gene. From the original 159 colonies, eleven colonies demonstrated >175 times more cells in the fourth (top) decade of fluorescence compared with analogous colonies grown in the presence of 5-FOA. These eleven colonies were further evaluated by selectively isolating the pJG4-5 plasmids that contained cDNA library members. This was accomplished by transformation of this *TRP1*-containing plasmid into the auxotrophic *E. coli* strain, KC8. The isolated pJG4-5 plasmids were retransformed into yeast that carried the tribrid system, and fluorescent reporter gene expression was reassessed by flow cytometry. These experiments revealed that one of the eleven isolated plasmids significantly affected reporter gene expression. The AD fusion protein encoded by this library plasmid was sequenced and analyzed with the on-line program BLAST[19] and its homology with human genes was assessed. The plasmid was found to encode the full-length protein tyrosine kinase Fyn.

Fyn is a functional tyrosine kinase when expressed in yeast

Fyn protein tyrosine kinase ($p59^{fyn}$) is a member of the Src family of kinases and propagates signal transduction pathways that control the survival of T-lymphocytes.[38,39] Hence, the gene that encodes Fyn represents a likely PTK target for isolation from a T-lymphocyte-derived cDNA library. To further verify that the gene encoding this PTK was required for the activation of the fluorescent reporter, omission control experiments were carried out as shown in Figure 4 D–G. These experiments confirmed that all elements of the yeast tribrid system (Fyn, LexA-Y_4 substrate, and B42–SH2 adapter protein) were critical for significant activation of reporter gene expression. Furthermore, the fluorescent reporter gene was not activated by Fyn when the Y_4 of the universal substrate were substituted with four phenylalanine resides (F_4). This confirms the importance of this amino acid in the substrate (Figure 4 H). Additional confirmation of the tyrosine kinase activity of Fyn was obtained by immunoblotting of yeast extracts (Figure 5). These experiments demonstrated that Fyn phosphorylates the universal PTK substrate in living yeast cells.

Conclusion

We have demonstrated that a yeast tribrid system that is equipped with a potentially universal alanine-rich substrate and a B42–SH2 adapter protein, can provide a novel tool for the functional cloning of genes that encode PTKs. To validate this strategy, we used FACS to clone the gene that encodes the Src-family kinase Fyn from a T-lymphocyte-derived cDNA library. This approach could provide a valuable tool for the iden-

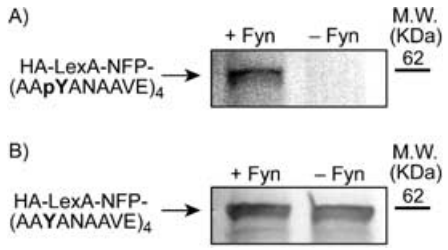

Figure 5. Intracellular phosphorylation of the universal substrate by Fyn kinase expressed in yeast. A) Phosphotyrosine was detected with an anti-phosphotyrosine IgG. B) The substrate fusion-protein was independently identified with an anti-HA IgG against a fused epitope tag. After induction of protein expression for 4 h, each lane was loaded with equivalent amounts of extract from ca. 2×10^7 cells.

tification, characterization, and evaluation of enzymes involved in a variety of protein post-translational modifications.

Experimental Section

General: Standard techniques for plasmid construction[40] and yeast transformation[41] were employed. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA), restriction endonucleases and T4 DNA ligase were from New England Biolabs, *Pfu* polymerase was from Stratagene, Pt *Taq* polymerase was from BD Biosciences, and DNA purification kits from Qiagen. Microbiological media (Luria broth, Brent Supplement Mixtures (BSM), yeast nitrogen base, carbon sources, M9 salts, yeast extract, and peptone) were from Difco and qBiogene (Irvine, CA). Prepoured solid yeast-selection media was purchased from KD Medical (Columbia, MD). DNA sequences were confirmed by automated dideoxynucleotide sequencing at The Pennsylvania State University Nucleic Acid Facility. Flow cytometry data was acquired at The Pennsylvania State University Center for Quantitative Cell Analysis.

Bacterial and yeast strains: *E. coli* DH5-α (Invitrogen) was employed for plasmid construction. *E. coli* KC8 (BD Biosciences) was used to rescue pJG4-5-derived yeast plasmids that carried the *TRP1* selection marker. *S. cerevisiae* FY250 (*MATα*, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*)—a gift from Prof. M. Ptashne—was employed for yeast tribrid assays.

Plasmid construction: The fluorescent reporter vector, pDCLryEGFP, combines the four *colE1* operators (eight LexA binding sites) upstream of the *GAL1–GAL10* divergent promoter region[29–31] with *yEGFP*. These control regions from pSH18-34 were amplified by PCR (primers: 5′ KpnI-LexAop: 5′-AGGGGTACCGACAGGTTATCAGCAACAACA-3′; 3′ EcoRI-Gal1(1–29): 5′-ATCGAATTCGCACTTTTCGGCCAATGGTCT-3′). The primers replace the upstream *Eco*RI site with a *Kpn*I site and preserve the downstream *Eco*RI site. This cassette was incorporated into *Eco*RI/*Kpn*I digested pBC103[32]. This is a YEplac181[42] derivative that contains a 2-micron origin, *LEU2* selection marker, *Eco*RI/*Xho*I multiple cloning site (MCS), and a downstream *ADH1* transcription terminator. The *yEGFP* gene was amplified by PCR from plasmid pSVA17[43] (a gift from S. Avery) with flanking 5′ *Eco*RI and 3′ *Xho*I sites. It was then inserted directly downstream of the LexA binding sites into the *Eco*RI/*Xho*I digested MCS, to provide pDCLryEGFP.

Plasmids pAMLexA2-NFP-$(AAYANAA)_4$ and pAMLexA2-NFP-$(AAFANAA)_4$ were used to express the universal PTK substrate and the phenylalanine mutant negative control. These plasmids encode the nonfluorescent Y66F GFP mutant (NFP) as a spacer protein but are otherwise identical to previously reported substrates.[22] The NFP was prepared by PCR megaprimer mutagenesis[44] from a *GFPuv* gene (Clontech), which is modified to lack internal *Mfe*I, *Xho*I, and *Sal*I restriction sites. The primers were: 5′ EcoRI-ATG-GFP: 5′-CAGGAATTCATGAGTAAAGGAGAAGAACTTTTC-3′, 3′ GFP (Y66F): 5′-GGAAAAGCATTGAACACCAAAAGAGAAAGTAGTGCAAG-3′, and 3′ XhoI-GFP (-M233): 5′-CGGAGTCTCGAGCATGCCATGTGTAATCCCAGCAGC-3′.

Plasmid pDCUAD, bearing a *URA3* selection marker, was used to express the B42–SH2 fusion protein. This plasmid was constructed by ligation of the *Kpn*I/*Bam*HI fragment from pJG4-5[37] into *Kpn*I/*Bam*HI-digested pRS426.[45] pJG4-5 contains the *GAL1* promoter, HA epitope tag, B42-AD, SV40 nuclear localization signal, MCS (*Eco*RI/*Xho*I), and *ADH1* terminator. Ligation of a previously described Grb2-SH2 cassette[22] into *Eco*RI/*Xho*I digested pDCUAD afforded vector pDCUAD-Grb2-SH2.

Positive controls analogous to PTKs encoded by the cDNA library, previously reported to be catalytically active fragments of PTKs v-Abl (237–630) and v-Src (137–526),[22] were digested with *Eco*RI/*Xho*I and ligated into similarly digested pJG4-5. The control plasmid, pAM423-B42–LexA, which expresses this strong transcriptional activator, has been previously described.[23]

Screening the cDNA library by FACS: *S. cerevisiae* FY250 yeast were transformed with three plasmids: pAMLexA2-NFP-$AAYANAA_4$, pDCUAD-B42–Grb2-SH2, and pDCLryEGFP. These yeast were plated on solid yeast media (BSM ura$^-$, his$^-$, leu$^-$, 2% glucose) and incubated at 30 °C for 3–4 days to allow for colony growth. Multiple combined colonies harboring the tribrid-system plasmids were transformed with a commercial Jurkat T-lymphocyte cDNA library (OriGene Technologies, Rockville, MD) by using a lithium acetate/polyethylene glycol high-efficiency transformation method.[46] The transformation mixture was directly resuspended in liquid yeast media (10 mL, BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% glucose) and incubated with shaking (30 °C, 30 h) to allow for selective growth of transformants. Cells from this culture (5 mL) were harvested by centrifugation (4300 rpm, 5 min), resuspended in noninducing liquid media (10 mL, BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% raffinose) and incubated with shaking (aerobic conditions, 30 °C, 16 h). Expression of tribrid-system components and cDNA library members was induced by adding galactose to the liquid media (10 mL, BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% galactose, 1% raffinose) at cell density OD_{590} ~0.4. The cells were further incubated with shaking for 4.5 h at 30 °C.

The resulting culture was analyzed by flow cytometry, and subjected to FACS by using an EPICS Elite flow cytometer (Beckman–Coulter) equipped with Expo software. Living cells were identified by forward-scatter and side-scatter dot plots, and yEGFP fluorescence was measured by excitation at 488 nm. A 550 nm dichroic long pass filter split the emission, sending the green light to PMT2 (photomultiplier 2), which was interfaced to an additional 525 nm bandpass filter. The sensitivity of the flow cytometer was attenuated to provide minimal (< 10 out of 15000) events (cells) in the top decade of fluorescence for an omission control experiment, in which no PTKs were expressed. When compared to a positive control experiment, in which PTKs v-Abl (237–630) or v-Src (137–526) were expressed, the ratio of fluorescent events in the top decade was about 1:6000 (omission control:PTK expressed). Yeast from the cDNA library that activated reporter gene expression in this fourth (top) decade of fluorescence were isolated by FACS into omni-trays (Nunc) that were filled with solid yeast media (BSM ura$^-$, trp$^-$,

his$^-$, leu$^-$, 2% glucose). The plates were incubated at 30 °C for 3–4 days to allow for colony growth. These colonies were replica plated onto omni-trays filled with solid yeast media (BSM trp$^-$, his$^-$, leu$^-$, 2% glucose, 0.1% 5-FOA) and incubated at 30 °C for an additional 4–5 days to remove the pDCUAD-Grb2-SH2 plasmid. Comparative analyses by flow cytometry of transformants cultured from these two plates separated false positives (i.e., yeast exhibiting significant fluorescence in the absence of the B42–SH2 component) from putative PTK hits. Yeasts from the master plates with >175 times more counts in the top decade of fluorescence than plates containing 5-FOA were considered to be "hits". The pJG4-5 library plasmid was isolated from these hits. The cDNA library members were sequenced and these sequences were compared with known human genes by using on-line BLAST programs.[19] Colonies that lacked B42–SH2 and exhibited no events were assigned a value of 1 for the calculation.

Fluorescent reporter gene assays: The yeast tribrid system was assayed essentially as described previously,[22] but flow cytometry was used to quantify reporter gene expression. Briefly, yeast transformants harboring the appropriate plasmids were incubated (30 °C, 16 h) in liquid media (BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% raffinose) with shaking. At $OD_{590}\sim0.4$, the cultures were resuspended in galactose-containing liquid media (BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% galactose, 1% raffinose) to induce protein expression. After further incubation (4 h, 30 °C) fluorescence was quantified by flow cytometry.

Analysis by flow cytometry: The flow cytometry data shown in Figure 4 was obtained with a Beckman–Coulter XL-MCL benchtop flow cytometer equipped with a 15 mW air-cooled argon-laser. Fluorescence was detected by excitation at 488 nm, splitting the emission with a 550 nm dichroic, and collecting emitted photons through a long pass filter. Fluorescence measurements were conducted with living cells, as determined by forward-scatter and side-scatter dot plots. Histograms were generated to represent the fluorescence properties of 15000 living cells. The flow cytometer PMT-FL1 settings were typically maintained between 725–800 so as to retain fewer than ten events in the top decade of fluorescence for omission-control experiments (i.e., yeast transformed with empty plasmids).

Isolation of plasmids from yeast: Yeast transformants identified as hits were incubated (30 °C, 16 h) in liquid selection media (4 mL, BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% glucose). Plasmid DNA was isolated by glass bead lysis followed by miniprep purification by using commercially available spin columns (Qiagen). Chemically competent *E. coli* KC8 (BD Biosciences) were transformed with this yeast plasmid and cells harboring the pJG4-5 vector were selected by incubation (37 °C, 16 h) on solid media (minimal M9 medium supplemented with SC Trp$^-$ (BD Biosciences), ampicillin (50 mg mL^{-1}), thiamine-HCl (1 M), and 1% glucose). The pJG4-5 plasmid containing the cDNA insert was isolated and the insert was sequenced.

Immunoblotting: Yeast were transformed with the tribrid-system plasmids and pJG4-5-Fyn or empty pJG4-5 as a negative control. These transformants were grown for 16 h in liquid media (BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% raffinose), resuspended in protein induction media (10 mL, BSM ura$^-$, trp$^-$, his$^-$, leu$^-$, 2% galactose, 1% raffinose), and further incubated with shaking (30 °C, 4 h) to give an $OD_{590}\sim0.4$. The cell pellet was isolated by centrifugation (10 min, 4300 rpm), washed with water (1.0 mL), and resuspended in sample buffer (0.1 mL).[47] The tube containing this yeast suspension was frozen at −80 °C (10 min) and subsequently placed in boiling water (10 min). This cell lysate was cleared by centrifugation (14000 rpm, 2 min), treated with the Compat-Able protein assay-reagent set (Pierce), and resuspended in sample buffer (0.1 mL).[47] Equivalent volumes of lysates were analyzed in duplicate by SDS-PAGE (15% Tris-glycine, Cambrex, Rockland, ME) followed by semidry transfer (Hoefer, San Francisco, CA) of proteins to nitrocellulose (Pall Life Sciences, East Hills, NY). The nitrocellulose membrane was divided and probed independently with two different primary antibodies. Mouse anti-phosphotyrosine IgG alkaline phosphatase conjugate (Southern Biotechnology Associates, Inc., Birmingham, AL) was used to detect phosphotyrosine. Mouse anti-HA IgG (Sigma) as a primary antibody and rabbit anti-mouse alkaline phosphatase conjugate (Sigma) as a secondary antibody, were used to verify the identity of the HA-tagged universal substrate (LexA-NFP-AAYANAA$_4$). The alkaline phosphatase conjugates were visualized with Western blue stabilized alkaline phosphatase substrate (Promega).

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