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Rapid Detection of Protein Tyrosine Kinase Activity in Recombinant Yeast Expressing a Universal Substrate

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Abstract: Yeast two-hybrid systems are powerful proteomics tools for the discovery of protein−protein interactions. However, these systems are typically unable to detect interactions dependent on post-translational modifications such as tyrosine phosphorylation. We report a novel yeast tribrid system that expresses a potentially universal protein tyrosine kinase (PTK) substrate to detect diverse PTKs. Validation with the oncogenic kinases v-Abl and v-Src, which exhibit divergent substrate specificities, demonstrated significant potential for cloning PTKs en masse from cDNA libraries.

Keywords: molecular recognition • signal transduction • phosphotyrosine • Grb2 • SH2 • LexA • B42 • GFP

Yeast two-hybrid systems are powerful proteomics tools for the identification and characterization of protein−protein interactions.[1] Yet, a significant drawback of traditional yeast two-hybrid assays concerns their inability to detect protein−protein interactions dependent on post-translational modifications such as tyrosine phosphorylation.[2] The identification of protein−protein interactions initiated by protein tyrosine kinases (PTKs) is particularly important because PTKs play critical roles in the regulation of signal transduction cascades controlling diverse cellular functions including cellular proliferation, differentiation, and apoptosis.[3,4] Furthermore, many PTKs are major drug targets. For example, members of the Abl and Src families of PTKs are responsible for the proliferation of several types of cancers.[3]

Yeast tribrid systems have been reported that detect interactions between tyrosine-phosphorylated proteins and cognate Src homology-2 (SH2) phosphotyrosine-binding domains.[2,5−14] These systems typically reconstitute protein−protein interactions dependent on tyrosine phosphorylation by co-expressing three functional protein components. These components include the PTK of interest, a DNA-bound "bait" protein bearing the tyrosine-containing peptide substrate, and a "prey" protein that binds phosphotyrosine and is linked to an activation domain. Fruitful PTK-mediated protein−protein interactions between bait and prey result in recruitment of the transcriptional machinery to DNA enhancer or promoter sequences controlling the expression of a reporter gene. As a consequence, yeast tribrid systems have significant potential for proteomics studies directed at the discovery of novel PTKs from cDNA libraries. However, PTK enzymes are known to exhibit distinct substrate specificities,[15−18] and these specificities limit the utility of yeast-based assays to identify novel PTKs.

We report here the construction of a novel yeast genetic system designed for the discovery of diverse PTK enzymes. To detect kinases that phosphorylate diverse peptide sequences, a potentially universal PTK substrate was constructed. This substrate bears the peptide sequence AA**Y**ANAA (Figure 1) and comprised the "bait" protein in the yeast tribrid system. This peptide was designed on the basis of the known recognition epitope of PTKs, which predominantly encompasses residues −2 to +4 surrounding the key tyrosine (Y).[15−18] Furthermore, asparagine (N) was incorporated at the +2 position of the substrate to enhance the affinity of the phosphopeptide product for the Grb2 SH2 domain.[19,20] This SH2 domain comprised the "prey" protein of the tribrid system and was incorporated to detect tyrosine phosphorylation by activating reporter gene expression (Figure 2). Alanine residues (A) were incorporated flanking the key tyrosine of the substrate to eliminate both favorable and unfavorable interactions beyond the β-carbon in an attempt to broaden the accessibility of the substrate to diverse tyrosine kinases.

To engineer yeast for the discovery and characterization of novel PTKs, genes encoding three protein components were expressed in *S. cerevisiae* (Figure 2). To validate this approach, catalytically active fragments of v-Abl and v-Src were chosen for investigation. These enzymes comprise viral nonreceptor PTKs that induce the uncontrolled proliferation of murine leukemia[21] and avian sarcoma[22] cells. These kinases were investigated because their substrate specificities differ substantially. For example, v-Abl preferentially phosphorylates the hydrophobic substrate sequence VI**Y**AAPF,[16,17] whereas v-Src optimally phosphorylates the highly acidic sequence EI**Y**GEFD.[16,17] Moreover, previous in vitro studies have shown that v-Src poorly phosphorylates an optimal v-Abl substrate peptide.[17] Conversely, v-Abl poorly phosphorylates optimal v-Src substrate sequences.[17]

To investigate whether the alanine-rich substrate shown in Figure 1 might be phosphorylated by diverse tyrosine kinases expressed in yeast, genes encoding this peptide sequence were expressed fused to green fluorescent protein (GFP), which was linked to the bacterial LexA DNA binding domain. The GFP protein was employed as a spacer to display the substrate to the kinase because highly efficient kinase substrates have been previously linked to the GFP C-terminus.[23] The LexA protein component forms strong noncovalent interactions with LexA DNA sites upstream of the reporter gene encoding β-galactosi-

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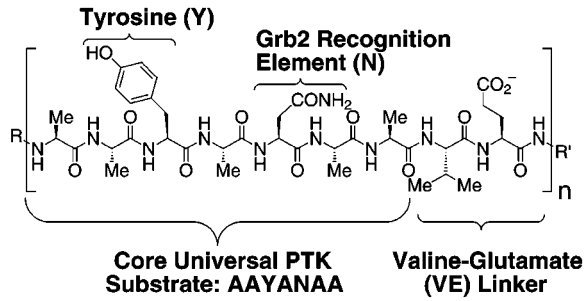

Figure 1. Structural depiction of the universal PTK substrate sequence AA**Y**ANAA.

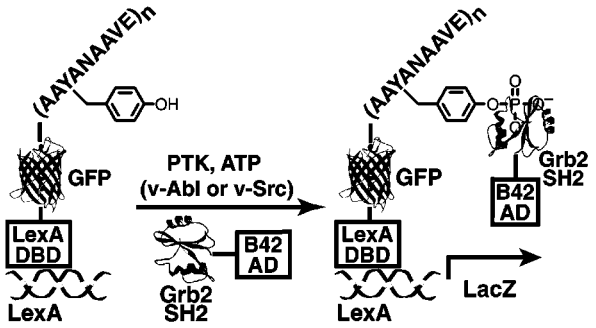

Figure 2. Schematic depiction of the yeast tribrid system incorporating a universal PTK substrate. Expression of the β-galactosidase (*LacZ*) reporter gene is controlled by coexpression of three protein components: (1) the PTK, which catalyzes phosphoryl transfer from ATP to protein tyrosine residues; (2) the multimeric universal tyrosine-containing substrate linked to GFP and fused to the LexA DNA binding domain (DBD); and (3) the Grb2 SH2 domain fused to the B42 activation domain (AD).

dase. The Grb2 SH2 domain fused to the bacterial B42 activation domain (AD) was coexpressed to activate reporter gene expression upon binding to the phosphorylated peptide substrate. Coexpression of a PTK was predicted to conditionally activate expression of the reporter gene by phosphorylating tyrosine residues of the substrate peptide and enabling recognition of the substrate by the Grb2 SH2 domain. This combination of strong noncovalent interactions was designed to recruit components of the transcriptional machinery to DNA and activate expression of β-galactosidase.[24,25]

Effective yeast hybrid assays typically require high-level protein expression to substantially activate reporter gene expression.[24,25] However, the phosphorylation of endogenous yeast proteins by overexpressed PTKs can affect yeast growth.[26] To circumvent potential problems associated with PTK expression, all protein components were placed under the control of the galactose-inducible *GAL1* promoter on multicopy episomal plasmids.[24,25] This approach enabled growth of yeast under noninducing conditions; galactose was subsequently added to induce protein expression for analysis.

Initial yeast tribrid experiments examined only a single copy of the AA**Y**ANAA substrate linked to LexA-GFP. However, this single-copy yielded only 4-fold (v-Abl) or insignificant (v-Src) increases in reporter gene expression (Figure 3). Fortuitously, analogous experiments employing dimeric or tetrameric analogues of this peptide substrate were found to significantly enhance reporter gene expression over basal levels (Figure 3). Remarkably, despite their highly divergent sequence specificities, expression of both v-Abl and v-Src substantially activated

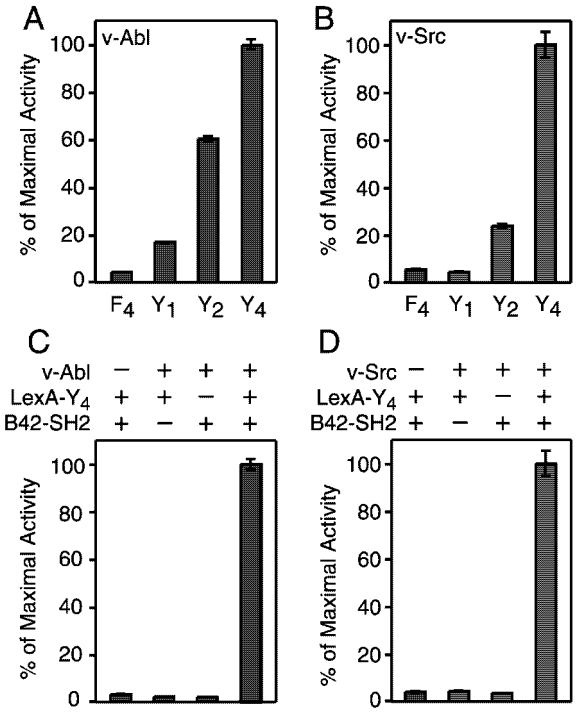

Figure 3. Analysis of reporter gene expression in the yeast tribrid system. Panels A and B: coexpression of v-Abl (panel A) or v-Src (panel B), B42-Grb2 SH2, and LexA-GFP-(AA**X**ANAA)$_n$. X corresponds to either phenylalanine (F) or tyrosine (Y). The subscript indicates the number of copies of the substrate (Y_n) or control (F_n) fused to LexA-GFP. Panels C and D: omission control experiments. Protein components were omitted by transformation with empty vectors. Expression of v-Abl in the Y_4 assay conferred ~75% greater overall transcriptional activity compared with v-Src. Values shown reflect the mean of three experiments.

reporter gene expression. Comparison of the putative universal substrate (AA**Y**ANAA)$_4$ with a corresponding control sequence substituting tyrosine for phenylalanine (AA**F**ANAA)$_4$ revealed that the tyrosine-containing peptide enhanced reporter gene expression by 24-fold (v-Abl) or 18-fold (v-Src) (Figure 3). Control experiments confirmed that all elements are critical for activation of gene expression (Figure 3). Furthermore, immunoblotting confirmed the expression of all protein components and the production of PTK activity in yeast (data provided in the Supporting Information).

Yeast hybrid assays are powerful proteomics tools that enable screening of cDNA libraries to discover previously uncharacterized proteins. The yeast tribrid assay described herein incorporates a potentially universal protein tyrosine kinase substrate designed to facilitate the discovery of diverse members of this class of enzymes. Enzymes potentially amenable for identification by this method include both nonreceptor protein tyrosine kinases and catalytically active intracellular fragments of receptor tyrosine kinases. Given the importance of these enzymes in human disease, this strategy may enable the identification of novel drug targets and advance our understanding of PTKs in signal transduction pathways.

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Supporting Information Available: Experimental procedures, immunoblots, and details regarding plasmid construction. This material is available free of charge via the Internet at http://pubs.acs.org.

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