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Enzyme-linked enzyme binding assay for Pin1 WW domain ligands

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Abstract

Peptidyl prolyl cis-trans isomerase (PPIase) interacting with NIMA-1 (Pin1) catalyzes the cis-trans isomerization of pSer/pThr-Pro amide bonds. Pin1 is a two-domain protein that represents a promising target for the treatment of cancer. Both domains of Pin1 bind the pSer/pThr-Pro motif; PPIase enzymatic activity occurs in the catalytic domain, and the WW domain acts as a recognition module for the pSer/pThr-Pro motif. An assay we call an Enzyme-Linked Enzyme Binding Assay (ELEBA), was developed to measure the K_d of ligands that bind selectively to the WW domain. A ligand specific for the WW domain of Pin1 was covalently immobilized in a 96-well plate. Commercially available Pin1 conjugated to horseradish peroxidase was used for chemiluminescent detection of ligands that block the association of the WW domain with immobilized ligand. The peptide ligands were derived from the cell cycle regulatory phosphatase, Cdc25c, residues 45-50. The K_d values for Fmoc-VPRpTPVGGGK-NH₂ and Ac-VPRpTPV-NH₂ were determined to be $36 \pm 4 \mu\text{M}$ and $110 \pm 30 \mu\text{M}$ respectively. The ELEBA offers a selective approach to detect ligands that bind to the Pin1 WW domain, even in the presence of the catalytic domain. This method may be applied to any dual specificity, multi-domain protein.

Keywords

Pin1; WW domain; PPIase; HRP; chemiluminescent binding assay; Pin1 ligands

Pin1 is a two-domain protein that catalyzes the cis-trans isomerization of pSer/pThr-Pro motifs through its PPIase (Peptidyl Prolyl Isomerase) catalytic domain [1;2]. Pin1 also contains a WW domain, so called because it has two invariant Trp residues [3]. The WW domain binds pSer/pThr-Pro motifs in target ligands, but does not catalyze cis-trans proline isomerization [1;3]. The Pin1 WW domain interaction with ligands occurs through a conserved phosphate-binding pocket, present in Pin1 and its homologues [1;4]. The Pin1 WW domain binds and regulates phosphorylated proteins at different stages of the cell cycle [4;5;6], and the WW domain is the site of Pin1 regulation by protein kinase A [7]. Pin1 phosphoprotein binding activity is abolished by phosphorylation of Ser16, located in the WW domain [7]. Peng and coworkers have shown that a Pin1 ligand from Cdc25, EQPLpTPVTDL, affects the dynamics of Pin1 side chains at the hydrophobic interface between the WW domain and the catalytic domain [8]. The possibility of a tight-binding WW domain ligand that blocks Pin1 association with its

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physiological substrates represents an alternative approach for inactivation of Pin1, a potential strategy for cancer therapy {Xu, 2009 #485}.

In previous efforts to identify Pin1 WW domain ligands with prospective clinical applications, several focused compound libraries have been screened by a variety of methods: fluorescence anisotropy, Nuclear Magnetic Resonance (NMR), Förster resonance energy transfer (FRET), and virtual screening [4;9;10;11;12]. However, for screening larger libraries, these techniques have various limitations, such as the requirement for fluorescent labeling of ligands, the amount of protein needed, the use of isolated WW domain, isotopic labeling of protein, or the time for data acquisition and interpretation [4;9;10;11;12]. We now describe an assay to screen for ligands that bind to the Pin1 WW domain. The Enzyme-Linked Enzyme Binding Assay (ELEBA) is simple to use compared with other protein-ligand interaction techniques. This assay can be adapted to screen compound libraries with multiple domains, or multimeric proteins with different ligand affinities and specificities in each domain or protein sub-unit, such as proteins containing Src homology (SH2 and SH3) domains [13], phosphotyrosine-binding (PTB) domains, or other protein-interacting modules. The major requirement for the assay is to identify a selective ligand with moderate affinity, but high specificity, for the site of interest. This is already known for the Pin1 WW domain [4].

For the ELEBA, we synthesized peptides containing the VPRpTPV core of the dual-specificity phosphatase Cdc25c at the pThr48–Pro49 position. Fluorescence anisotropy has shown that the Pin1 WW domain is highly selective for the VPRpTPV sequence, with an affinity of 7.7 μ M for the WW domain, and no detectable binding to the catalytic domain [4]. Synthetic H–GGGGVPRpTPVGG–NH₂ was covalently immobilized to a 96-well microtiter plate, and commercially available Pin1 (residues 2–163) conjugated to horseradish peroxidase (Pin1–HRP) was used for chemiluminescent detection of binding. Full-length Pin1 is more stable than the isolated WW domain [4]. WW domain ligands in solution that have similar or better affinity than the immobilized ligand block the binding of the WW domain with the immobilized ligand on the plate. Therefore, WW domain ligands in complex with Pin1–HRP are removed during washing, and produce little or no colorimetric signal after the addition of peroxidase substrate.

Materials and Methods

Peptide Synthesis

Peptides were synthesized using manual solid-phase peptide synthesis (SPPS) in disposable polypropylene columns (Thermo Scientific) with standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. All Fmoc amino acids were purchased from Novabiochem. 4-Methylbenzhydrylamine (MBHA, Rink amide) resin (100 mg, 0.66 meq/g, 0.064 meq) was swelled in CH₂Cl₂ (3 mL) for 20 min, and the Fmoc protecting group was removed with 20% piperidine in *N*-methyl-2-pyrrolidone (NMP) (2 $\times$ 3 mL). The resin was washed twice with NMP (ca. 8 mL). The first amino acid in the sequence (3 eq.) was coupled to the resin using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (70 mg, 0.19 mmol), *N*-hydroxybenzotriazole (HOBt) (29 mg, 0.19 mmol), and diisopropylethylamine (DIEA) (67 μ L, 0.38 mmol) in NMP (2 mL), and the mixture was double coupled (2 $\times$ 2 h). Completion of coupling was monitored by the Kaiser test for primary amines, and by the *p*-chloranil test for prolines [14;15]. The reaction mixture was washed with NMP (2 $\times$ 6 mL), and a solution of acetic anhydride (0.2 mL, 1.1 mmol), DIEA (0.2 mL, 2.1 mmol), and CH₂Cl₂ (1.6 mL) was added and shaken for 10 min to cap any uncoupled amines. The Fmoc group was removed with 20% piperidine in NMP (2 $\times$ 3 mL). The following amino acids were added one at a time in the same manner: Fmoc–Gly–OH (59 mg, 0.19 mmol), Fmoc–Val–OH (65 mg, 0.19 mmol), Fmoc–Pro–OH (65 mg, 0.19 mmol), Fmoc–Thr(PO₂H(OBzl))–OH (98 mg, 0.19 mmol), Fmoc–Lys(Boc)–OH (93 mg, 0.19 mmol), and Fmoc–Arg(Pbf)–OH (125

mg, 0.19 mmol), Fmoc-Leu-OH (68 mg, 0.19 mmol), and Fmoc-Ser(tBu)-OH (74 mg, 0.19 mmol). The peptide resins were cleaved with a mixture of 95% CF₃CO₂H, 2.5% H₂O, 2.5% triisopropylsilane for 4 h at 30 °C. With exceptions noted, crude peptides were precipitated with cold diethyl ether (3 × 10 mL), and purified using reverse phase high-pressure liquid chromatography (RP-HPLC), using a Waters C18 XBridge™ 5 μm, 19 × 100 mm semi-preparative column (unless otherwise noted) on a Varian Pro-Star 218 HPLC with linear gradients as noted, flow rate 10 mL/min, λ = 210 nm. CH₃CN was removed under vacuum, and the water was removed by lyophilization. Analytical HPLC were obtained on a Beckman HPLC with linear gradients as noted, flow rate 1.0 mL/min, λ = 210 nm. Peptides were characterized by ¹H NMR on an INOVA-400 MHz, and by Liquid Chromatography-Mass Spectrometry (LC-MS) on an Agilent 1100 LC-ThermoFinnigan TSQ MS with electrospray ionization (ESI+) for molecular ion identification (Supplementary Material).

H-GGGGVPRpTPVGG-NH₂ 1 was purified by RP-HPLC with 0.1% ammonium formate in 5% to 60% CH₃CN/H₂O gradient over 7 min. The desired peak was collected at 6 min to give 8 mg (11% yield). Analytical HPLC using a Waters C18 XBridge™ 2.5 μm, 4.6 × 50 mm column, with 5% CH₃CN/H₂O for 2 min, then 5% to 50% gradient over 15 min showed a major peak at 14.2 min, 91.2% pure. LC-MS 13.25 min, calcd. for C₄₂H₇₄N₁₆O₁₆P [M + H]⁺ *m/z* = 1089.5, found *m/z* = 1089.7 (Supplementary Material, Figs. S1-S5).

Fmoc-VPRpTPVGGGK-NH₂ 2 was purified by RP-HPLC with 0.1% CF₃CO₂H in 20% to 70% CH₃CN/H₂O gradient over 8 min. The desired peak was collected at 6.5 min to give 38 mg (47% yield). Analytical HPLC using an XBridge™ BET130 3.5 μm C18 4.6 × 50 mm column, with 5% CH₃CN/H₂O for 2 min, then 5% to 80% gradient over 5 min, showed a major peak at 4.3 min, 96.2% pure. LC-MS 14.04 min, calcd. for C₅₇H₈₇N₁₅O₁₆P [M + H]⁺ *m/z* = 1268.6, found *m/z* = 1268.7 (Supplementary Material, Figs S6-S9).

Ac-VPRpTPV-NH₂ 3 was purified by RP-HPLC with 5% to 60% CH₃CN/H₂O gradient over 18 min. The desired peak was collected at 11 min to give 9 mg (18% yield). Analytical HPLC using a Waters C18 XBridge™ 2.5 μm, 4.6 × 50 mm column, with 5% CH₃CN/H₂O for 2 min, then 5% to 60% gradient over 5 min, then 60% for 4.5 min, showed a major peak at 5.48 min, 91.7% pure. LC-MS 15.32 min, calcd. for C₃₂H₅₈N₁₀O₁₁P [M + H]⁺ *m/z* = 789.4, found *m/z* = 789.4 (Supplementary Material, Figs S10-S13).

Ac-LPTPLSPS-NH₂ 4 was purified by RP-HPLC, using a Varian Polaris 10 μm, C18 100 × 212 mm semi-preparative column, with 0.1% CF₃CO₂H in 10% to 60% CH₃CN/H₂O gradient over 15 min, λ = 220 nm. The desired peak was collected at 7 min to obtain 9.4 mg (16% yield). Analytical HPLC using a Varian Polaris 5 μm C18 4.4 × 100 mm column, with 0.1% CF₃CO₂H in 10% to 40% CH₃CN/H₂O gradient over 15 min, showed a major peak at 8.5 min, 84.5% pure. LC-MS 14 min, calcd. for C₄₄H₇₃N₁₀O₁₃ [M + H]⁺ *m/z* = 949.5, found *m/z* = 949.6 (Supplementary Material, Figs S14-S17).

Immobilization of ligands to the 96-well plate

H-GGGGVPRpTPVGG-NH₂, ligand **1**, (100 μL, 2.8 mM) in phosphate buffered saline (PBS): 15 mM sodium phosphate, 2 mM potassium phosphate buffer, and 150 mM NaCl at pH 9.2, was added to each well of a DNA-BIND™ microtiter plate (Corning Life Sciences, Wilkes Barre, PA). The plate was sealed with clear tape (Corning Costar Corporation), and incubated for 2 h at 37 °C with shaking. The uncoupled ligand was decanted and the wells were washed (3 × 150 μL/well). The wash buffer contained 10 nM imidazole, and 0.05% Tween-20 in PBS at pH 7.3. The plate was shaken in air and inverted on a paper towel until dry ca. 30 min, and the remaining active sites were blocked with PBS buffer containing 5% sucrose, 0.05% NaN₃, 2% bovine serum albumin (BSA), Fraction V, ELISA grade (CALBIOCHEM) at pH 7.3 (100 μL/well). The plate was sealed with tape, and incubated overnight at 0 °C. The solution

was decanted via pipet, and the plate was rinsed 3 times with a squeeze bottle of wash buffer, and inverted on a paper towel to drain excess buffer.

Fmoc-VPRpTPVGGGK-NH₂, ligand **2**, (100 μ L, 0.1 mM) was covalently attached to the 96-well plate through the ϵ -amino group of the Lys side chain at pH 11.0, otherwise as described above.

ELEBA procedure

Competitive binding with the Pin1 WW domain of ligands **2** and **3** with plate-linked GGGGVPRpTPVGG-NH₂ **1** was measured. UV-Vis spectroscopy was used for accurate determination of ligand **2** concentration using the Fmoc ϵ value of 3981 cm⁻¹ M⁻¹ at 256 nm. Ligand **3** was dried over P₂O₅ for 2 days prior to assay, weighed (4.3 mg) to determine the concentration, and dissolved in 200 mM phosphate, 30 mM NaCl, pH 7.0 (1.0 mL, 5.4 mM). Recombinant human Pin1-HRP (R&D Systems, Minneapolis, MN) was diluted to a final concentration of 200 pM in PBS buffer containing 2% BSA at pH 7.3 (Fig S18). For ligand **2**, the concentrations were: 1.1, 3.3, 7.7, 15, 44, 95, 146, 230, 460, and 730 μ M; for ligand **3**: 2, 6, 12, 25, 40, 120, and 180 μ M; for ligand **4**: 7.7, 95, 460, 1000, 2000 μ M; for ligand **5**: 7.7, 95, 460 μ M; and for ligand **6**: 7.7, 95, 460, 1000 μ M. For ligand **2** against ligand **2** linked to the plate, the concentrations were: 1.1, 4.0, 10, 33, 62, 102, 345, and 550 μ M.

The Pin1-HRP solution was incubated at room temperature for 1 hour with orbital shaking in the plate with ligands **2** - **6** in a total volume of 100 μ L/well. The solutions were decanted, and the wells were washed with wash buffer (3 $\times$ 150 μ L/well). Hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB, R&D Systems, Minneapolis, MN) were added to the plate in a 1:1 ratio (100 μ L/well), and incubated at room temperature for 20 minutes in the dark. The stop solution, 2 N H₂SO₄ (50 μ L/well) was added to the 96-well plate. The optical density (OD) of each well was determined immediately, using a plate reader (Spectra Max 340 PC) with UV absorbance detection at 450 nm. The 96-well plate with covalently attached ligand is reusable after appropriate washing steps and storage (Supplementary Material, Fig. S19).

Results

Design, synthesis, and immobilization of ligand **1**

The Cdc25c-T48 peptide of Verdecia et al.[4] was modified with the addition of two glycine residues at the C-terminus to avoid diketopiperazine formation [16], and four glycine residues at the N-terminus as an additional spacer between the ligand and the 96-well plate surface to give H-GGGGVPRpTPVGG-NH₂ **1**. Computer modeling was used to evaluate the interactions of ligand **1** with the Pin1 WW domain binding pocket. All of the glycine residues extended outside of the Pin1 contact region (Supplementary Material, Fig. S20).

Peptides were synthesized using standard Fmoc chemistry on a Rink amide resin to produce amidated C-termini. During purification of phosphopeptides, improved recovery from HPLC was obtained without acid in the mobile phase, which we have observed to cause β -elimination of phosphate from pSer or pThr. Ligand **4**, that is not phosphorylated, was purified with CF₃CO₂H in the mobile phase.

ELEBA validation with conjugated Pin1-HRP

Ligand **1** was covalently bound to the 96-well plate, which contained an *N*-oxysuccinimide ester (NOS) activating group that was reacted with the peptide primary amine at pH 9.2 to capture the ligand covalently on the solid surface (Fig. 1) [17]. The effect of the buffers we used in the ELEBA assay were tested at pH values $\pm$ 1 of their respective pK_a values, and with and without 150 mM NaCl (Supplementary Material, Figs. S21 and S22). For the ELEBA, the

most suitable buffer to prevent Pin1–HRP non-specific binding with the plate surface was PBS at pH 7.3 with 150 mM NaCl. Two specific WW domain ligands Fmoc–VPRpTPVGGGK–NH₂ **2** and Ac–VPRpTPV–NH₂ **3** were used as positive controls for ELEBA validation (Table 1). Commercially available recombinant human Pin1, residues 2 to 163, chemically conjugated via a peptide linker to horseradish peroxidase (Pin1–HRP) was used with varying concentrations of **2** and **3** to measure affinity (Fig. 2). As the concentrations of **2** and **3** were increased, they competed with ligand **1** on the plate for binding to the Pin1 WW domain, leaving unbound Pin1–HRP which was washed away.

The data for ligands **2** and **3** were fitted to a dose-response binding curve, using Kaleidagraph v. 4.3:

$$\% \text{ of binding} = a + \frac{(b - a)}{(1 + 10^{(c - \log(L))})} \quad \text{Eq. 1}$$

where [L] is the concentration of the ligand in solution. The dose-response binding curve measures the binding constant at a single concentration of Pin1–HRP in the presence of varying concentrations of competing ligands **2** and **3** [18]. We normalized the data to % Pin1–HRP bound to the plate-linked ligand by setting the lowest concentration of competitive ligand in solution to 100% binding. The top of the curve is a plateau represented by the value of a. The highest concentration of ligand in solution was set to 1% binding, which is represented by the bottom of the curve as a plateau, corresponding to the value of b. The log of the concentration at zero is undefined. Instead, we entered very low competitor concentration values of 1.1 μM and 2 μM for ligands **2** and **3** respectively. The c value corresponds to the response halfway between a and b values. This variable is sometimes called ED₅₀ (effective dose, 50%), or IC₅₀ (inhibitory concentration, 50%). The competitive K_d values were calculated by solving for log [L] when % binding (y) = 50%. The values: a = 85 ± 2, b = −2.6 ± 3, and c = 1.7 ± 0.07 with R² = 0.9966, were fitted for ligand **2**. The K_d value of 36 ± 4 μM was determined from three data sets (Table 1, Fig. 2). The values: a = 108 ± 6, b = −29 ± 20, and c = 2.0 ± 0.2 with R² = 0.9877, were fitted for ligand **3**. The K_d value of 110 ± 30 μM was determined from three data sets (Table 1, Fig. 2).

ELEBA selectivity for WW domain ligands

To study the specificity of the assay for phosphopeptides, an unphosphorylated, proline-rich peptide, c-Myc 56-64, Ac–LPTPPLSPS–NH₂ **4**, was assayed. Pin1 is known to regulate turnover of transcription factor c-Myc that is dependent on phosphoThr-58 [19]. Varying concentrations of **4** were used as described above. Ligand **4** was not able to compete with plate-linked ligand **1**, even at a concentration of 2 mM, and Pin1–HRP remained fully bound to the plate after washing (Fig. 3).

To further investigate the site of ligand binding to Pin1, two of our Pin1 PPIase inhibitors, Fmoc–pSer–ψ[CH₂N]–Pro–2-(3-indolyl)ethylamine **5**, and Ac–pSer–ψ[(Z)CH=C]–Pip–2-(2-naphthyl)ethylamine **6** [20;21], were assayed. Both PPIase catalysis inhibitors were unable to prevent Pin1 WW domain binding with ligand **1**, even at high concentrations (Table 1 and Fig. 3). Non-specific binding of Pin1–HRP to the plate surface was reduced by blocking any residual NOS sites with the non-interfering protein, BSA. The affinity of Pin1–HRP for bound ligand **1** was tested in the presence and absence of ligand **1** attached to the 96-well plate surface. In the presence of ligand **1**, Pin1–HRP was detected at high levels on the plate after washing (OD 1.2 – 2.7). However, in the absence of ligand **1**, only 0.89 – 2.5% non-specifically bound Pin1–HRP was detected on the plate after washing (Fig. 3).

The effect of the plate-linked ligand in the competitive ELEBA

Fmoc-VPRpTPVGGGK-NH₂, ligand **2**, was covalently attached to the 96-well plate via the Lys side chain ε-amine. Pin1-HRP was incubated with different concentrations of ligand **2** in solution, in competition with ligand **2** bound to the plate. The variable values fitted were: $a = 99 \pm 3$, $b = -10 \pm 4$, and $c = 1.8 \pm 0.1$ with $R^2 = 0.9976$. The K_d value of $66 \pm 10 \mu\text{M}$ was determined from two data sets (Fig. 4).

Discussion

Pin1 contains catalytic (PPIase) and regulatory (WW) domains within the same polypeptide chain. Both domains have specificity for the pSer/pThr-Pro motif, but with different binding affinities and specificities [4;5]. The study of ligand-binding site preference with full-length Pin1 is challenging because high concentrations of ligands that bind at the Pin1 catalytic site can mask binding to the Pin1 WW domain. Therefore, the isolated domains are often used in the identification of ligand-binding-site preference [4;11;12].

We designed the Enzyme-Linked Enzyme Binding Assay (ELEBA) to selectively identify ligands that bind at the Pin1 WW domain using full-length Pin1 (Fig. 1). The ELEBA was intended to measure only Pin1 WW domain affinities, not PPIase inhibition constants. The availability of Pin1-HRP conjugated protein allowed us to develop a high-throughput assay, similar to an ELISA, without the requirement for antibodies. Such covalent protein-HRP conjugates have been used to date in Western blots with Protein A and Protein G HRP conjugates [22], and in a viral protease assay with Gag precursor substrate-HRP conjugate [23]. The labeled peptide sequence from Cdc25c 45-50, rhodamine-VPRpTPV, is highly selective for the WW domain ($7.7 \mu\text{M}$), with no detectable binding to the PPIase domain [4]. We used peptides with the sequence VPRpTPV covalently linked to 96-well plates for the selective identification of ligands that compete for the Pin1 WW domain.

Glycine residues were added at either the N- or the C-termini of ligands **1** and **2**, with the objective of extending the peptide at least 12 or 15 atoms away from the 96-well plate surface to avoid Pin1-HRP absorption on the plate surface (Fig. 1). Computer modeling indicated that the additional Gly residues do not affect Pin1 binding with the VPRpTPV core (Fig. S20), in agreement with published results that the WW domain phosphate-binding pocket is capable of interacting with up to five consecutive amino acid residues, and polar residues do not affect the peptide affinities [4;11].

Binding affinities for ligands **2** and **3** with the Pin1 WW domain were measured by the ELEBA in a concentration-dependent manner (Table 1, Fig. 2). Both ligands **2** and **3** effectively competed with ligand **1** on the plate surface for the Pin1 WW domain. The affinity of Pin1-HRP for Fmoc-VPRpTPVGGGK-NH₂ **2** was 3-fold tighter than for Ac-VPRpTPV-NH₂ **3** (Table 1). A similar binding affinity has been measured for EQPLpTPVTDL ($120 \mu\text{M}$), lacking a hydrophobic label, by NMR [8;9;10]. Since ligand **3** exhibited weaker affinity than ligand **2** for the Pin1 WW domain, the Fmoc group contributed to the binding affinity. The Fmoc derivative **2** was more similar in hydrophobicity to the original rhodamine-VPRpTPV ligand used in the fluorescence assay, which may explain the higher affinity [4]. This demonstrates the utility of the ELEBA in differentiating the affinities of two similar ligands.

Fmoc-VPRpTPVGGGK-NH₂ **2** showed a 2-fold tighter Pin1-HRP binding affinity in competition with ligand **1** covalently linked to the plate, than it did in competition with ligand **2** covalently linked to the plate (Fig. 4). This also suggests that the hydrophobic Fmoc group of ligand **2** improves the affinity for the Pin1 WW domain in comparison with ligand **1** [4; 11]. Either ligand **1** or ligand **2** attached to the plate gives a suitable assay for screening libraries to obtain hit compounds with affinity for the WW domain in the micromolar or better range.

The ELEBA was developed as a method for high-throughput screening, rather than as an absolute measure of binding affinity. Since our method is competitive with ligand linked to a plate, it is not to be taken as a direct measurement of binding affinity. We chose to compare the affinities of several different ligands primarily to permit flexibility in the choice of plate-linked ligand. In some cases, it makes more sense to have a weak ligand on the plate so that a high-throughput assay would not miss weaker potential hits. On the other hand, if the high-throughput assay gave too many hits, it might be necessary to make the screening more stringent with a tighter binding ligand bound to the plate.

An unphosphorylated, proline-rich peptide, Ac-LPTPPLSPS-NH₂, showed no detectable binding to the Pin1 WW domain, in agreement with published data that the Pin1 WW domain does not interact with unphosphorylated peptides [3;4]. Two of our small molecule inhibitors of the Pin1 catalytic activity, **5** and **6**, respectively were used as negative controls [20;21]. Neither compound showed any competition with ligand **1**, indicating that the inhibitors were unable to block the Pin1 WW domain binding site, and that Pin1-HRP was fully capable of interacting with ligand **1** covalently linked to the plate (Table 1). These experiments demonstrated the selectivity of the ELEBA for phosphorylated ligands that bind to the WW domain.

Conclusions

The ELEBA is a non-catalytic binding assay for the identification of ligands that bind to the Pin1 WW domain. The assay is fast, selective, and amenable to screening ligand libraries, with the potential for automation. The assay is advantageous over existing methods, because it selectively identifies compounds that bind to the Pin1 WW domain, it uses small quantities of reagents, and no antibodies, fluorescent labels, or radioactive labels are required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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a. Peptide-surface coupling

b. ELEBA Scheme

Fig. 1.

ELEBA methodology, a) At alkaline pH, the free N-terminus of H-GGGGVPRpTPVGG-NH₂ **1** was coupled to an *N*-oxysuccinimide ester becoming covalently linked to the plate surface. b) Pin1-HRP conjugated enzyme bound to immobilized ligand **1** in the absence of ligands that block the WW domain-binding site. The amount of Pin1-HRP bound to the plate is measured by oxidation of the HRP-substrate. (Prepared with ChemBioDraw Ultra and Canvas™ 8.)

Fig. 2. Binding curves showing the % of Pin1–HRP bound to plate-linked GGGGVPRpTPVGG–NH₂ **1** at varying concentrations of ligands **2** and **3**. Data are an average of $N = 3$ with standard deviations shown in the error bars; the data were fitted to equation 1, using KaleidaGraph 4.3. Left: binding curve for ligand **2**. Right: binding curve for ligand **3**. The calculated K_d values were $36 \pm 4 \mu\text{M}$ for **2** and $110 \pm 30 \mu\text{M}$ for **3**. (Prepared with Kaleidagraph 4.3.)

Pin1-HRP binds WW domain ligands selectively

Fig. 3.

Pin1-HRP was incubated with varying concentrations of ligands 2 - 6, to detect the ability of the ligands to block Pin1 WW association with immobilized ligand 1. Pin1-HRP binding with ligand 1 on the plate decreased with increasing concentration of 2 in solution. Ligands 4, 5, and 6 did not show any decrease in binding of Pin1-HRP to the plate. The last column shows Pin1-HRP nonspecific binding (NSB) when ligand 1 was not linked to the plate. Mean of $N = 3$ was used for data analysis with standard deviations shown as error bars. (Prepared with Kaleidagraph 4.3.)

Fig. 4.
The effect of plate-linked ligand **2** in the competitive ELEBA. The K_d value for Pin1–HRP was calculated to be $66 \pm 10 \mu\text{M}$ for $N = 3$ with standard deviations shown as error bars. (Prepared with Kaleidagraph 4.3.)

Table 1

Binding affinities (K_d) for ligands used in ELEBA. The pT/S-P motif is shown in bold.

Ligand	Structure	K_d	Pin1 PPIase IC ₅₀
1	H-GGGGVPR p TPVGG-NH ₂	N.A. ^a	N.A. ^a
2	Fmoc-VPR p TPVGGGK-NH ₂	36 ± 4 μM	N.A. ^a
3	Ac-VPR p TPV-NH ₂	110 ± 30 μM	N.A. ^a
4	Ac-LPTPPL SPS -NH ₂	> 2 mM ^b	N.A. ^a
5	Fmoc- pS -Ψ[CH ₂ N]-P-2-(3-indolyl)ethylamine	> 0.5 mM ^b	6.3 ± 0.4 μM [19]
6	Ac- pS -Ψ[(Z)CH=C]-Pip-2-(2-naphthyl)ethylamine	> 1 mM ^b	141 ± 18 μM [20]

^a Not applicable.

^b No detectable binding.