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Rapid screening of protein–protein interaction inhibitors using the protease exclusion assay

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ABSTRACT

We have previously developed a sensitive and modular homogenous biosensor system using peptides to detect target ligands. By transposing the basic mechanistic principle of the nuclease protection assay into this biosensor framework, we have developed the protease exclusion (PE) assay which can discern antagonists of protein–protein interactions in a rapid, single-step format. We demonstrate the concept with multiple protein–peptide pairs and validate the method by successfully screening a small molecule library for compounds capable of inhibiting the therapeutically relevant p53–Mdm2 interaction. The Protease Exclusion method adds to the compendium of assays available for rapid analyte detection and is particularly suited for drug screening applications.

1. Introduction

It has been shown that more than 80% of proteins do not exhibit activity in the absence of complex formation, indicating the importance of protein–protein interactions in fundamental cellular processes (Berggard et al., 2007; Veselovsky et al., 2002). This observation has led to the relatively new endeavor of seeking antagonists of protein–protein interactions for therapeutic purposes (Arkin and Wells, 2004). Notable successes include numerous small molecule and peptidic antagonists of the p53–Mdm2 interaction (Shangary and Wang, 2009; Zhao and Bernard, 2013), SM-164 (inhibitor of the XIAP-caspase interaction) (Sun et al., 2008) and ABT-737 (blocks the interaction between Bcl-XL and Bak) (Parrondo et al., 2013).

A large number of these protein–protein interactions are mediated by specialized modular protein domains like PDZ, SH2, and SH3, which bind to cognate peptides in their respective interaction partners (Beuming et al., 2005; Pawson et al., 2001; Petsalaki and Russell, 2008). In addition to the widespread usage of specialized peptide binding domains, it is estimated that in more than 50% of globular protein–protein interactions, the dominant contribution from one protein of the interacting pair can be reduced to a single peptide (London et al., 2010). Similarly, Jochim and Arora (2010) have analyzed the PDB structural database and shown that helical peptide segments form a major constituent of a number of protein–protein interactions which could be susceptible to small molecule inhibitors. Large numbers of peptide mimotopes, which can mimic one binding partner of a protein–protein interacting pair, have been discovered, typically using peptide phage display (Huang et al., 2012). Numerous databases and tools have been created to enable facile study of peptide–protein interactions (Shtatland et al., 2007; Vanhee et al., 2010). These facts point to the salience of peptide–protein interactions in the protein–protein interaction network. It would thus be very useful from a therapeutic perspective if novel methods could be developed to enable rapid and facile screening of drugs which can disrupt peptide–protein interactions (Chen et al., 1993). Prevailing methods such as Enzyme-linked immunosorbent assay (ELISA), Surface plasmon resonance (SPR) and Fluorescence polarization (FP) can be adapted to study protein–protein interaction and inhibitor screening. However, none of these methods fulfill all the criteria desirable for drug screening, such as a homogenous set-up for facile high throughput screening, absence of washing and/or immobilization steps, robustness in the presence of a wide variety of autofluorescent small molecule drugs, presence of serum, cell lysates and other complex fluids, and a turn-on instead of a turn-off signal in response to an inhibitor. For example, ELISA and SPR are time and labor intensive, whilst Fluorescence Polarization is a turn-off method which can suffer interference from autofluorescent drugs or small metabolites (Owicki, 2000). Other homogenous methods such as the protein fragment complementation assay (PCA) typically give a turn-off signal in response to interaction inhibitors and require the fusion of split protein domains to the interacting proteins (Hashimoto et al., 2009). Therefore, in order to meet the requirements of high throughput screening and accelerate the detection of antagonists of protein–protein interaction with high sensitivity and minimal process time, designing a homogenous screening system with high specificity

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and robustness is necessary. We have therefore developed such a method by extrapolating the principles of the nuclease protection assay into a protein based system. We demonstrate the method using both fluorescence and enzyme-coupled readout formats. We further validate it in a small-molecule (fragment) screen for inhibitors of the p53–Mdm2 interaction, where it demonstrated high sensitivity and specificity compared with other methods.

2. Materials and methods

Chemicals and reagents were purchased from Sigma Aldrich, unless indicated otherwise. The Mdm2 protein used here is derived from N terminal domain (amino acids 18–125) of wild type Mdm2 protein and engineered with 10 × His tag, the protein was expressed in *E. coli* and purified by immobilized affinity chromatography as described earlier (Brown et al., 2011b). The eukaryotic translation initiation factor 4E (eIF4E) was produced as previously reported (Brown et al., 2007). All synthetic peptides used were obtained from Bio-Synthesis, Inc (USA).

2.1. Experimental verification using fluorescence labeled peptide

The PE concept was first validated by using a peptide (P1) labeled with a fluorophore and quencher pair. The peptide sequence is as follows:

E(EDANS)–SG DDDDR-GK (Dabcyl)-TSFAEYWNLLSP-GS.

In this peptide, the fluorophore 5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) is conjugated as a side chain to glutamic acid followed by amino acids SG as a linker. DDDDR is an enhanced enterokinase cleavage recognition site (Boulware and Daugherty, 2006). The EDANS quencher 4-(dimethylaminoazo)benzene-4-carboxylic acid (Dabcyl) is conjugated to the side chain of lysine followed by a high affinity peptide sequence (TSFAEYWNLLSP) derived by phage display which binds the p53 binding pocket in the Mdm2 N-terminal domain. In a final volume of 25 μl with 4% DMSO buffered by phosphate buffered saline (PBS) at pH 7.3, 1.33 μM fluorescent labeled peptide (E(EDANS)–SG DDDDR GK (Dabcyl) TSFAEYWNLLSP GS), 152 pM enterokinase and 3.2 μM Mdm2 are present. Apart from these constant reagents, triplicates of varying concentrations of Nutlin, wild type p53 peptide (ETFSDLWKLLS) and a mutant p53 peptide with critical residues mutated to alanine (ETASDLAKLAP) were added to the reaction. Enterokinase was added last, after a 5 min delay to ensure that no premature cleavage of peptide occurs. The resulting reaction was read on a Perkin Elmer plate reader in a 384 well black bottom plate (Greiner) with excitation at 335 nm and emission at 490 nm. Readings were taken every 5 min. The signal was calculated as the difference between emission intensity at reading 3 and 5. Every experiment was repeated three times and the average with standard error was reported.

2.2. Plasmid construction and oligonucleotides

Four plasmids were used in this study. HA–enterokinase plasmid was constructed by inverse PCR (Nirantar et al., 2013) of a codon optimized Tem1–BLIP (D49A) cassette (Genscript) placed in the NdeI XhoI sites of pET28a using the oligonucleotides 1 and 2 as described in Supplementary Table 1. Oligo 1 is a Tem1 reverse oligo whose 5′ partially codes for the intended linker sequence. Oligo 2 is a BLIP forward oligo whose 5′ has 15 bases complementary to oligo 1 for infusion cloning purposes, and codes for the rest of the peptide linker sequence. After the inverse PCR, the PCR product was treated with DpnI, purified and treated using the infusion cloning enzyme (Clontech) to enable intra-molecular infusion, followed by transformation in JM109 HIT competent cells (RBS Biosciences). The HA–TEV plasmid was made as above, using oligonucleotides 3 and 4 (Supplementary Table 1), using the HA–enterokinase plasmid as a template.

The Mdm2–enterokinase sensor was constructed in the same way, except that the Tem1–BLIP (D49A) template used has a linker present between Tem1 and BLIP. Oligonucleotide 5 (Supplementary Table 1) is a reverse oligo, while tandem oligonucleotides 6 and 7 are forward oligos complementary to the linker sequence. Tandem oligonucleotides were used due to the length of the peptide linker to be inserted. The eiF4E–enterokinase sensor was made as the Mdm2–enterokinase sensor, using oligonucleotide 5 as a reverse oligo and oligonucleotides 8 and 9 as tandem forward oligos (Supplementary Table 1).

2.3. Sensor protein production

The relevant plasmid was transformed into SHuffle T7 Express Competent *Escherichia coli* cell (New England Biolabs), the transformed cells were grown overnight in LB medium containing 50 μg/ml kanamycin sulfate at 30 °C under shaking conditions. 1% (v/v) of the overnight culture was inoculated into 250 ml LB medium at 30 °C, and protein expression of the fusion proteins was induced with IPTG at OD600 = 0.7–0.8. After 4 h post-induction at room temperature, the cells were harvested by centrifugation. The washed cell pellets were resuspended in 20 ml 50 mM Phosphate buffer (pH 7.4) and disrupted by sonication using a digital sonifier (Life Technologies, Novex). Sonication was performed for 15 cycles at 5 s/cycle, followed by 10 s cooling after each sonication cycle. The lysed cells were centrifuged at 10,000*g* for 30 min, and the suspension was then collected. The filtered supernatant of cell lysate containing sensor protein with a 6 × His tag was loaded onto a 1 ml Ni Sepharose His Trap column (GE Healthcare). The column was pre-equilibrated with Buffer A (50 mM phosphate buffer, 300 mM NaCl, 30 mM imidazole, pH 7.4). After washing with 10 column volume (CV) of Buffer A, the target sensor protein was eluted at 80% Buffer B (phosphate buffer, 300 mM NaCl, 500 mM imidazole, pH 7.4) over 15 CVs. The recovered sensor protein was stored in −80 °C for further usage.

2.4. HA–enterokinase sensor assay

All reactions were performed in 25 μl PBS with 250 μM substrate (Nitrocefin, Merck) and Greiner Bio 384 well transparent bottom plates were used to hold the reactions. The reaction was monitored using absorbance measurements at OD492 on a Perkin Elmer plate reader every 2 min. The sensor response to enterokinase was first investigated by mixing 5 nM relevant sensors (diluted from 1.25 μM stock) with various amount of enterokinase (0.3 nM–1.2 nM) at room temperature; TEV (1.2 nM) was used as the negative control. Subsequently, the sensor response to anti-HA antibody F-7 (Santa Cruz Biotech) was investigated by adding various amount of HA antibody (3 nM to 10 nM) in the presence of 1.2 nM enterokinase; 10 nM whole mouse IgG was used as the control. Sensor response to various concentrations of free HA peptide (0.8 nM to 8 μM) was carried out in the presence of 1.2 nM enterokinase and 10 nM anti-HA antibody, 8 μM p53 peptide was used as the control. The rate of substrate turnover, typically the OD492 value of read number 3 subtracted from that of read number 5 (OD492 readings were taken every 2 min) was denoted as the signal. Every experiment was repeated three times and the average with standard error was reported.

2.5. Mdm2–enterokinase sensor assay

The reaction was carried out in 25 μl PBS with 5 nM sensor, 0.15 nM enterokinase, 40 nM Mdm2 and 250 μM nitrocefin. Nutlin was used as the positive control and a mutant p53 peptide which cannot bind to Mdm2 was used as the negative control. The reaction was monitored using absorbance measurements at OD492 on a