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Genetic screen to dissect protein–protein interactions: ribonuclease inhibitor–ribonuclease A as a model system

Sang-Hyun Park[a,1] and Ronald T. Raines[a,b,*]

[a] *Department of Biochemistry, University of Wisconsin–Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA*
[b] *Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, WI 53706-1396, USA*

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Abstract

Protein–protein interactions are critical for the function of biological systems. Here, we describe a means to dissect a protein–protein interaction. Our method is based on the in vivo interaction between a target protein and the peptide epitopes derived from its partner. This interaction is detected by using hybrid proteins in which the target protein and peptide epitopes are fused to the DNA-binding domain of the lambda repressor protein. An interaction prevents the transcription of a reporter gene. The efficacy of this approach is demonstrated with the ribonuclease inhibitor protein and ribonuclease A, which form a complex with an equilibrium dissociation constant in the femtomolar range. Our method can enable the identification of residues important in a designated protein–protein interaction and the development of antagonists for that interaction.

Keywords: Cytotoxin; Epitope; Genetic screen; Lambda repressor; Protein–protein interaction; Ribonuclease A; Ribonuclease inhibitor

1. Introduction

Bovine pancreatic ribonuclease A (RNase A;[2] EC 3.1.27.5) is a 14-kDa protein that catalyzes the cleavage of single-stranded RNA. The biochemical and biophysical properties of RNase A are well characterized [1]. Although the biological role of RNase A itself is unknown, some homologs of RNase A are known to be cytotoxic [2].

The lack of cytotoxicity of RNase A itself is a consequence of its high affinity for the cytosolic ribonuclease inhibitor protein (RI [3,4]). Conversely, a key to the cytotoxicity of some RNase A homologs is their ability to evade RI [5]. RI is a 50-kDa modular protein consisting of leucine-rich repeats arranged symmetrically in a horseshoe shape [6–8]. RI forms a 1:1 complex with its target ribonucleases (Fig. 1A), and the equilibrium dissociation constant (K_d) of these complexes is in the femtomolar range [11,12]. RI inhibits the ribonucleolytic activity of RNase A by blocking access of an RNA substrate to the enzymic active site. Although the biological function of RI is unknown, RI is used widely to inhibit ribonucleolytic activity during numerous laboratory procedures, including in vitro translation [13] and cDNA synthesis [14].

A vast region of surface area (2551 Å^2) is buried in the interface of the RI · RNase A complex [6]. This interface is 60% larger than that of typical protease inhibitor-protease or antibody–protein antigen complexes [15]. The crystalline structure of the RI · RNase A complex provides atomic details of this intermolecular interactions. Still, which residues or segments of RNase A are most important for complex formation is unclear. Here, we describe a novel genetic screen to dissect a protein–protein interaction, and we demonstrate its utility with the RI · RNase A complex.

* Corresponding author. Fax: 1-608-262-3453.
E-mail address: raines@biochem.wisc.edu (R.T. Raines).

[1] Present address: Department of Cellular and Molecular Pharmacology, University of California, San Franscisco, CA 94143-0450, USA.

[2] *Abbreviations used:* cI, lambda repressor protein; IPTG, isopropyl-β-D-thiogalactopyranoside; NcI, N-terminal (DNA-binding) domain of lambda repressor protein; LB, Luria–Bertani; PCR, polymerase chain reaction; P-Gal, phenyl-β-D-galactopyranoside; RNase A, bovine pancreatic ribonuclease A; RI, ribonuclease inhibitor protein; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside.

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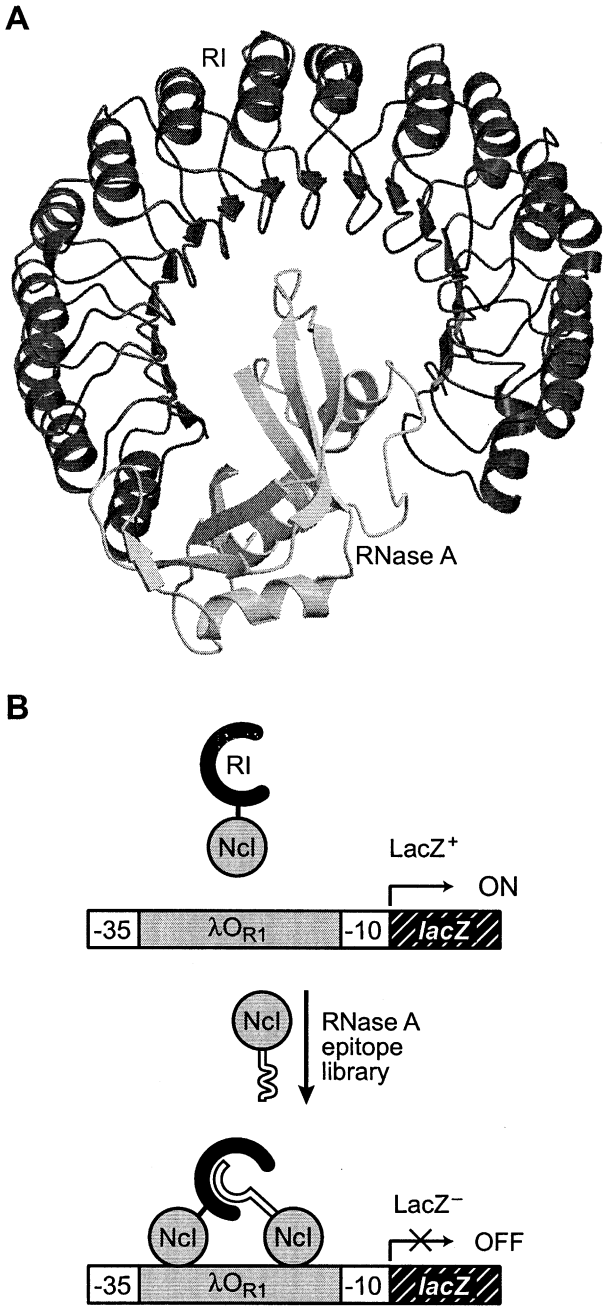

Fig. 1. (A) Three-dimensional structure of the complex between porcine ribonuclease inhibitor and ribonuclease A. Ribbon diagrams are created with the programs MOLSCRIPT [9] and RASTERS3D [10] by using coordinates derived from X-ray diffraction analysis (PDB entry 1DFJ [6]). (B) Basis for the genetic screen of an RNase A epitope library for segments that interact with RI. The NcI–RI fusion protein alone is not a functional repressor, and transcription of *lacZ* is not repressed (*lacZ*$^+$). Epitopes of RNase A are produced as a fusion to the C terminus of NcI. A functional repressor is reconstructed through binding of an epitope to RI and can be detected by screening for a *lacZ*$^-$ phenotype.

Our method takes advantage of the repressor protein (cI) from bacteriophage λ, which binds to its operator DNA as a dimer [16]. Previously, we used the lambda repressor system to identify inhibitors of a designated protein–protein interaction [17]. Others have used this system for related purposes [18–21]. Here, we use the lambda repressor system to examine the interaction between RI and peptides derived from RNase A. Our strategy is depicted in Fig. 1B. Briefly, a fusion protein is created in which RI is fused to the C terminus of the N-terminal (i.e., DNA-binding) domain of cI (NcI; residues 1–131). The resulting hybrid (NcI–RI) is not a functional repressor because RI does not form stable dimers. Next, a library of RNase A epitopes is fused to the C terminus of NcI. On concomitant production of NcI–RI and the NcI–epitope fusion proteins, a functional repressor is reconstructed via the interaction of RI with some RNase A epitopes. These epitopes are identified by a readily detectable phenotype that results from the repression of the transcription of a reporter gene, *lacZ*. This method enables the identification of residues important in a designated protein–protein interaction and the development of lead antagonists of that interaction.

2. Description of methods

2.1. General materials and methods

Materials. Escherichia coli strain MC1061 can be obtained from the American Type Culture Collection (Manassas, VA). Plasmid pACYC184 can be obtained from New England Biolabs (Beverly, MA). Plasmid pGEX-4T3, DNase I, dNTPs, Sephadex G-25 spin columns, and shrimp alkaline phosphatase can be obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Plasmid pGEM3Zf(+) and ΦX174 DNA/*Hin*fI markers can be obtained from Promega (Madison, WI). SYBR Green I can be obtained from Molecular Probes (Eugene, OR). *Pfu* DNA polymerase can be obtained from Stratagene (La Jolla, CA). All other enzymes for the manipulation of recombinant DNA can be obtained from either Promega or New England Biolabs.

Luria–Bertani (LB) medium contains tryptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L). Plates and top agar also contain agar (15 and 7 g/L, respectively).

The dissection of the RI–RNase A interaction required several specific reagents. Plasmid pBR–RI, which encodes human RI, was a generous gift from Promega. Plasmid pBXR, which encodes RNase A, was described by us previously [22]. Plasmids pJH370 and pJH391 and bacteriophage λKH54 were generous gifts of James C. Hu (Texas A&M University). Plasmid pJH370 directs the expression of NcI–zip, which is a fusion protein of NcI and a leucine zipper domain of the yeast transcription factor GCN4. Plasmid pJH391 is a "stuffer" plasmid for the construction of NcI fusion proteins, in which DNA encoding NcI is fused to a stuffer fragment (*lacZ*) for cloning purposes.

PCR. The PCR can be performed with *Pfu* DNA polymerase. All DNA fragments prepared by the PCR are sequenced and their identities are confirmed before subsequent subcloning steps.

Instruments. DNA sequencing can be performed with an ABI 377XL automated DNA sequencer from Applied Biosystems (Foster City, CA). Fluorescence gel scanning can be carried out with a fluorimager SI System from Molecular Dynamics (Sunnyvale, CA).

2.2. Reporter plasmid

Design. A reporter cassette (λP_R–*lacZ*–*rrnBT*$_2$) is constructed in which a *lacZ* gene is under control of a synthetic λP_R promoter containing a single copy of the λO_{R1} operator and is followed by an *rrnBT*$_2$ transcription terminator. This cassette was described previously [17]. The *tet* gene is removed from the original construct because screening of the epitope library does not require the selection for tetracycline resistance. The reporter cassette is inserted into a plasmid to generate a reporter plasmid, pSH58, which also directs the expression of NcI–RI (Fig. 2).

Construction. cDNA encoding human RI [23] is amplified from plasmid pBR–RI by the PCR using oligonucleotides P57 ACCTCGAGCCTGGACATCCA GAGC and P58 GAAGATCTTCAGGAGATGACC CTCAG, which contain *Xho*I and *Bgl*II sites (underlined), respectively. The resulting PCR fragment is inserted into the *Sal*I/*Bam*HI sites of plasmid pJH391 to create an expression vector for the NcI–RI fusion protein, pSH48.

A 653-bp *Hin*dIII/*Ssp*I fragment from pGEM3Zf(+) is ligated to a 2556-bp *Hin*dIII/*Hin*cII fragment from pACYC184. The resulting plasmid pSH46 is a general cloning vector containing a p15A replication origin, a chloramphenicol resistance marker, and multiple cloning sites from the pGEM3Z vector. A *Cla*I/*Bgl*II fragment from plasmid pSH48 encoding NcI–RI is ligated to a *Cla*I/*Bam*HI fragment of plasmid pSH46 to create plasmid pSH54. A DNA fragment containing the reporter cassette of λP_R–*lacZ*–*rrnBT*$_2$ is prepared by digesting plasmid pSH15 [17] with *Not*I and *Hin*dIII. The 5′ overhangs are filled in to blunt ends using the Klenow fragment and dNTPs. The resulting DNA fragment is inserted into the *Sma*I site of plasmid pSH54 to create plasmid pSH58 (Fig. 2). This plasmid directs the expression of NcI–RI in *E. coli*, as shown in Fig. 3.

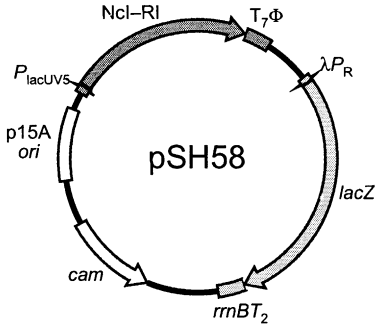

Fig. 2. Map of reporter plasmid pSH58. Plasmid pSH58 contains the reporter cassette (λP_R–*lacZ*–*rrnBT*$_2$) and the expression cassette for the NcI–RI fusion protein (P_{lacUV5}–NcI–RI-T7Φ). *rrnBT*$_2$ and T7Φ are transcription terminators from the *E. coli rrnB* ribosomal RNA operon and bacteriophage T7, respectively. Upon transformation with pSH58, *E. coli* cells show a *lacZ*$^+$ phenotype due to the inability of NcI–RI to repress the transcription of *lacZ* gene by the λP_R promoter.

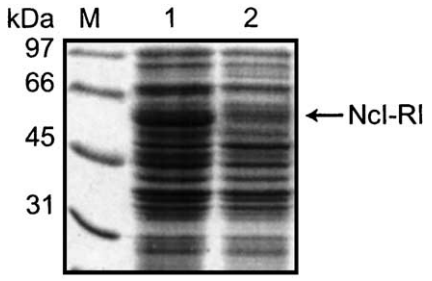

Fig. 3. SDS–PAGE analysis of the NcI–RI fusion protein. Lane M, molecular weight markers; lane 1, cellular extracts of pSH48/MC1061 induced with IPTG (0.5 mM); lane 2, cellular extracts of pSH48/MC1061 without induction.

Because epitope screening relies on the reconstruction of a functional repressor, it is important that NcI–RI alone neither bind to λO_{R1} nor act as a repressor. To test for this possibility, pSH58/MC1061 cells are subjected to a dot plaque assay. In this assay, cells producing a functional repressor undergo lysogenic growth upon infection with bacteriophage λKH54 [24], which has a deletion in the cI gene. Cells without a functional repressor undergo a lytic cycle upon infection with λKH54 and form plaques. λKH54 has been used as a tool to determine the existence of a repressor inside a cell [25,26]. As shown in Fig. 4, pSH58/MC1061 cells are sensitive to λKH54 infection, and therefore NcI–RI is capable of neither binding to λO_{R1} nor acting as a functional repressor.

2.3. Epitope library plasmid

Design. The overall scheme for the construction of an epitope library of RNase A fused to the C terminus of NcI is shown in Fig. 5. Briefly, a DNA fragment containing the open reading frame of RNase A is amplified by the PCR. This PCR product is subjected to partial digestion with DNase I in the presence of Mn^{2+} ion to generate small, blunt-ended DNA fragments (20–30 bp) that correspond to 7–10 amino acid residues. For the purpose of cloning, the ends of the digested DNA must be blunt. In the presence of Mg^{2+} ion, DNase I produces nicks in duplex DNA, and in the presence of Mn^{2+} ion, the enzyme produces double-stranded breaks in the DNA [27]. Reaction time and temperature are varied to

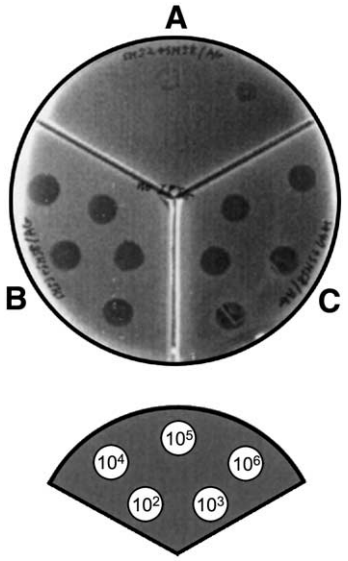

Fig. 4. Dot plaque assay of λKH54 infection of MC1061 cells producing NcI fusion proteins. Cells producing a functional repressor are immune to λKH54 infection. Cells that lack a functional repressor are sensitive to λKH54 and hence form plaques. Different amounts of λKH54 (10^2–10^6 pfu) are spotted on the surface of LB top agar containing cells to be tested. (A) MC1061 cells cotransformed with pSH52 and pSH58, so that the NcI–zip and NcI–RI fusion proteins are both produced. NcI–RI is not a functional repressor. (B) MC1061 cells cotransformed with pSH53 and pSH58, so that NcI and the NcI–RI fusion proteins are both produced. (C) MC1061 cells cotransformed with pSH47 and pSH58, where the NcI–RNase A and NcI–RI fusion proteins are both produced. Neither protein is a functional repressor, and RNase A cannot fold properly in the reducing environment of the *E. coli* cytosol.

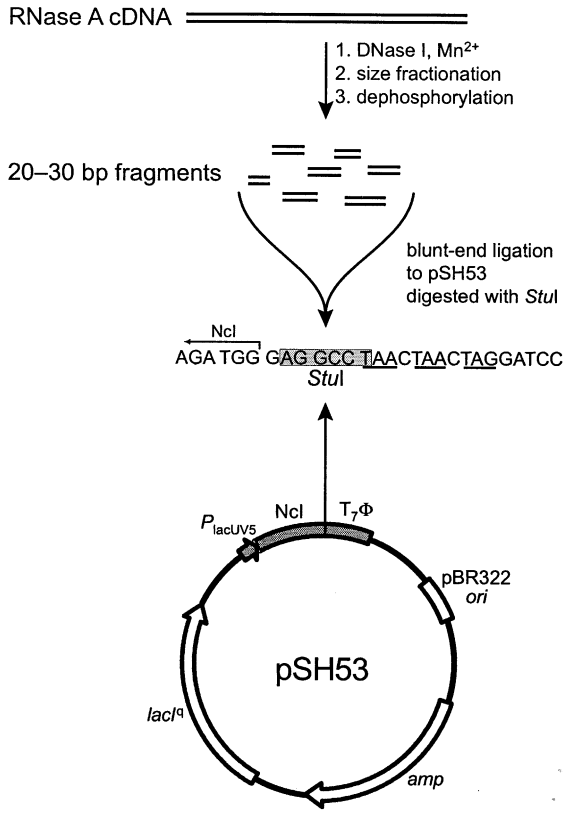

Fig. 5. Scheme for the construction of a plasmid that directs the expression of an RNase A epitope library fused to the C terminus of NcI. The size of this library is 5×10^5, and the average length of the epitopes is eight amino acid residues.

maximize the population of DNA in the desired size. DNA fragments with a size of 20–30 bp are recovered from a polyacrylamide gel (Fig. 6A), dephosphorylated, and ligated to *Stu*I-digested pSH53. Plasmid pSH53 has a unique *Stu*I site between the C terminus of NcI and three tandem repeats of stop codons (Fig. 5). The PCR-amplified cDNA of RNase A does not carry a *Stu*I recognition sequence. The ligation mixture is digested with *Stu*I to destroy any self-ligated pSH53 and to enrich selectively the plasmids that carry inserts.

Construction. Plasmid pGEX-4T3 is digested with *Bam*HI/*Bsu*36I, and the 5′ overhangs are filled in to blunt ends using the Klenow fragment with dNTPs. The DNA is purified from an agarose gel and made circular by ligation to create plasmid pSH49. This plasmid is identical to pGEX-4T3 except for the lack of an expression module for glutathione *S*-transferase. Plasmid pSH49 is cut with *Sal*I, the 5′ overhang is filled in to blunt ends, and the resulting DNA is digested with *Eco*RI. The *Eco*RI/*Eco*RV fragment from pJH370 is inserted into the blunt end/*Eco*RI fragment of pSH49 to create plasmid pSH52. The P_{lacUV5}–NcI fragment is amplified from pJH391 by the PCR using oligonucleotides P62 TTGGATCCTAGTTAGTT

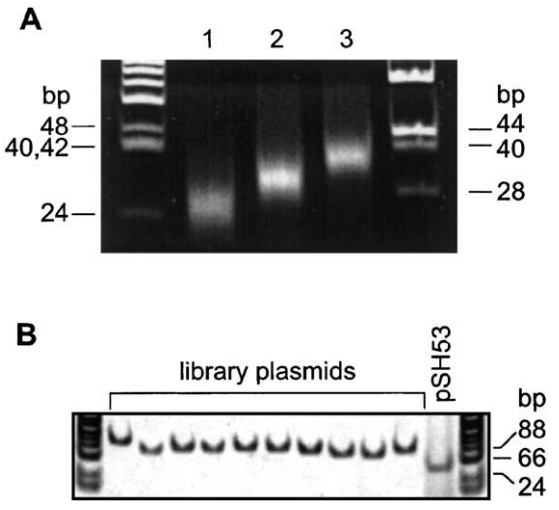

Fig. 6. (A) Analysis of DNA fragments for library construction. PCR-amplified cDNA encoding RNase A is digested partially by DNase I, and the resulting DNA is fractionated by size and purified from an acrylamide gel. DNA fragments of 20–45 bp are fractionated further into three groups. Lane 1, 20–30 bp, which is used for construction of the epitope library; lane 2, 28–35 bp; lane 3, 35–45 bp. (B) Analysis of epitope library plasmids by restriction digestion. Plasmids from 10 library clones and pSH53 are digested with *Bam*HI and *Hin*dIII, which flank the epitope insertion site. Digestion of the blank vector (pSH53) generates a 56-bp fragment. All 10 library plasmids carry inserts of the correct size (20–30 bp).

AGGCCTCCCATCTCTCCGCATCACCTTTG, which contains *Bam*HI and *Stu*I sites (underlined), and P63 TCGCTAGCCCCTTTCGTCTTCAAGAAT. The resulting PCR fragment is digested with *Eco*RI and *Bam*HI and inserted into the *Bam*HI/*Eco*RI sites of pSH52 to create an expression vector for the epitope library, pSH53.

The cDNA of RNase A is amplified by PCR using oligonucleotides P59 and P60, and the resulting 10 reaction mixtures are combined and purified. The PCR fragments are digested partially with DNase I by incubation in 0.20 mL of 50 mM Tris–HCl buffer (pH 7.5) containing $MgCl_2$ (10 mM), bovine serum albumin (50 μg/mL), $MnCl_2$ (10 mM), and DNase I (2 U) at 20 °C for 10 min. After incubation, EDTA (9 μL of a 0.5 M solution) and SDS [2 μL of a 10% (w/v) solution] are added to inactivate the DNase I. The resulting DNA is stained with SYBR Green I and subjected to electrophoresis along with fluorescein-labeled molecular weight markers in a polyacrylamide [10% (w/v)] gel in 1 × TBE buffer at 15 V/cm for 1.5 h. Immediately after electrophoresis, the gel is scanned with a fluorimager and DNA bands corresponding to 20- to 45-bp are cut out of the gel to fractionate the DNA into fragments of 20–30, 28–35, and 35–45 bp. DNA is purified from the gel pieces [28] and dephosphorylated using shrimp alkaline phosphatase.

The dephosphorylated epitope DNA inserts (20–30 bp; 0.20 μg) are ligated to *Stu*I-digested pSH53 (2 μg) using T4 DNA ligase with ATP by incubating at 37 °C for 6 h. The ligated DNA is digested with *Stu*I to linearize any self-ligated pSH53 and then desalted. One third of each DNA sample is transformed into electrocompetent XL-1 Blue cells as described previously [17]. Transformed cells are resuspended in SOC medium (1.0 mL) and grown at 37 °C for 1 h.

One fifth of the cells are subjected to library amplification, and the remaining cells are frozen and stored at −80 °C for future use. For amplification of the library, a cell culture (0.20 mL) is plated on a large petri dish of LB agar containing ampicillin (0.10 mg/mL) and grown at 37 °C for 16 h. LB medium (10 mL) is added to the plates, and cells are recovered by scrubbing the surface of the medium with a glass rod. The library plasmid DNA is prepared from the collected cells.

The size of the library created in this manner is 5×10^5, and >90% of the library plasmids carry an insert (Fig. 6B). The number of possible epitopes of RNase A with a length of eight amino acid residues is 117 ($= 124 - 8 + 1$). Considering the possible frames of codon triplets (3) and the possible directions (2) during a blunt-end ligation, the maximum size of the epitope library is 702 ($= 117 \times 2 \times 3$). The size of the epitope library (5×10^5) far exceeds this theoretical number (702). Thus, the library is likely to contain all possible epitopes.

2.4. Control plasmids

Control plasmids are constructed as negative and positive controls of the epitope library screening. Plasmid pSH53 (Fig. 5) directs the expression of NcI alone (vide supra). Plasmid pSH52 directs the expression of the NcI–zip fusion protein, which forms a dimer mediated by a leucine zipper domain [25,27]. Plasmid pSH47 directs the expression of NcI fused to intact RNase A, as reconstructed as follows.

DNA fragment containing NcI domain is amplified from plasmid pJH391 by PCR using oligonucleotides P50 GTATCTCGAGGCCCTTTCGTCTTC and P51 TTTCCTAGGTCGACACCCATCTCTCCGC, which contains a *Sty*I site (underlined). The PCR fragment is digested with *Eco*RI/*Sty*I and ligated to an *Eco*RI/*Spe*I fragment of pJH391 to create plasmid pSH50. A gene encoding wild-type RNase A is amplified from plasmid pBXR [22] using oligonucleotides P59 GCGTCGAC CAAGGAAACTGCAGC and P60 CGAGATCTACA CTGAAGCATCAAA, which contain *Sal*I and *Bgl*II sites (underlined), respectively. The PCR fragment is inserted into the *Sal*I/*Bam*HI sites of plasmid pJH391 to create an expression vector for the NcI–RNase A fusion protein, pSH47.

2.5. Epitope library screening

Epitope library plasmids (0.20 μg) and pSH58 (0.20 μg) are transformed simultaneously into electrocompetent MC1061 cells. Cotransformants are plated onto a large petri dish of LB–agar containing ampicillin (0.10 mg/mL), chloramphenicol (0.10 mg/mL), X-Gal (50 μg/mL), and IPTG (30 μM), and grown at 37 °C for 24 h until *lacZ*$^-$ (white) colonies appear. The library plasmids are rescued from *lacZ*$^-$ colonies and retransformed into *E. coli* strain XL-1 Blue.

2.6. Dot plaque assay

The initial positive clones are subjected to a dot plaque assay to confirm the formation of a functional repressor. The rescued library plasmids from *lacZ*$^-$ colonies are transformed again into pSH58/MC1061, and fresh overnight cultures are made in 2.0 mL of LB medium containing ampicillin (0.10 mg/mL), chloramphenicol (0.10 mg/mL), $MgSO_4$ (10 mM), and maltose (0.2% w/v). Cells (0.10 mL) are mixed with molten LB top agar (1.0 mL) containing ampicillin (0.10 mg/mL), chloramphenicol (0.10 mg/mL), and IPTG (30 μM) and poured onto an LB–agar plate with the same composition. After the top agar has dried, aliquots (3 μL) of bacteriophage λKH54 (containing 10^2–10^6 pfu) are spotted on the surface of the solid medium containing cells, and the resulting plates are incubated at 37 °C until plaques appear.

3. Sample results

MC1061 cells were transformed by electroporation with the library plasmids and pSH58. About 100 of 5000 cotransformants displayed a *lacZ*$^-$ phenotype (white colonies) in the presence of IPTG and X-Gal, and library plasmids were recovered from these *lacZ*$^-$ cotransformants. To test the ability of the epitopes from *lacZ*$^-$ clones to interact with RI, the library plasmids were retransformed into pSH58/MC1061 cells and the cotransformants were subjected to λKH54 infection. About 60 of the 100 *lacZ*$^-$ isolates showed immunity to λKH54 infection. The *lacZ*$^-$ and λKH54-immune clones were identified, and library plasmids from the clones were sequenced.

Sequence analysis of clones that showed immunity to λKH54 infection revealed five "hot spots" in the primary sequence of RNase A. These hot spots include residues 31–39, 68–74, 77–83, 99–104, and 108–114. Thus, these residues have demonstrable affinity for RI in vivo. Rigorous in vitro analyses that use actual peptides corresponding to these residues could reveal important insights into the RI–RNase A interaction. A correlation with analogous "hot spots" in RI, which have been identified by alanine-scanning mutagenesis [29,30], could also be enlightening.

4. Comments

Protein–protein interactions often bury a large interfacial surface area. Identifying those residues that are most important for the interaction is difficult, even with guidance from the three-dimensional structure of the protein–protein complex. Indeed, the surface area of a residue that is buried in a complex does not correlate with the energetic contribution of that residue to the interaction [31,32]. Traditionally, identifying the key residues has required time-consuming mutational analyses and extensive biochemical studies. The method described herein, which is based on a genetic screen, can facilitate this process.

We chose the RI · RNase A complex as a model system. We developed a genetic screen to identify epitopes in RNase A that interact with RI. This information could guide the creation of new variants of RNase A that evade RI and, hence, are potential cytotoxins [2,5]. Moreover, small-molecule antagonists of the RI–RNase A interaction could potentiate the cytotoxicity of existing cytotoxic variants.

The most difficult step in our method is the construction of the epitope library. This step relies on a blunt-end ligation of epitope DNA and the library plasmid (Fig. 3). To avoid concatenation, the epitope DNA is dephosphorylated prior to the ligation. The library plasmid, pSH53, is digested with *Stu*I to generate blunt ends. Intramolecular ligation (self-ligation of plasmid) is favored over intermolecular ligation (plasmid plus insert), especially when the inserts are dephosphorylated. It is then necessary to enrich the library in plasmids that carry an insert by eliminating self-ligated plasmids. Digestion with *Stu*I achieves this goal because only the self-ligated plasmid contains a *Stu*I site. Using this method, we are able to create an epitope library of RNase A with a size of 5×10^5 in which 90% of the plasmids carried an insert.

Considering the small size of RNase A (124 residues), linear epitopes longer than 10 residues would limit severely the diversity of the library and epitopes shorter than 6 residues would be unlikely to have sufficient binding affinity. Hence, the average size of epitopes is designed to be eight amino acid residues. Because the diversity of the epitope library is limited and the sequences in the library are restricted, it is unlikely for false positives that bind to NcI to occur. Thus, the initial *lacZ*$^-$ positives are not subjected to an extensive test for false positives. Instead, the interaction of RI and epitopes from *lacZ*$^-$ isolates is confirmed by an independent method based on λKH54 infection followed by a dot plaque assay. Non-false but irrelevant positives are likely to arise from those epitopes that happen to bind to a region of RI that differs from that in the RI · RNase A complex (Fig. 1A).

The method described herein can be expanded to the selection of a cDNA or random peptide library for ligands that have affinity to a protein of interest. Utilization of an appropriate substrate of β-galactosidase (e.g., P-Gal) and a host strain (e.g., *E. coli C*) allows for the counter selection for a *lacZ*$^-$ phenotype [33]. Then, reconstruction of a functional repressor through the interaction of the target protein and a ligand from the library would lead to cell growth. Such a selection would enable the facile screening of a large library ($>10^9$) for new protein–protein or protein–peptide interactions in vivo.

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