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Efficient isolation and elution of cellular proteins using aptamer-mediated protein precipitation assay

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ABSTRACT

Protein precipitation is one of the most widely used methods for antigen detection and purification in biological research. We developed a reproducible aptamer-mediated magnetic protein precipitation method that is able to efficiently capture, purify and isolate the target proteins. We discovered DNA aptamers having individually high affinity and specificity against human epidermal growth factor receptor (EGFR) and human insulin receptor (INSR). Using aptamers and magnetic beads, we showed it is highly efficient technique to enrich endogenous proteins complex and is applicable to identify physiologically relevant protein–protein interactions with minimized nonspecific binding of proteins. The results presented here indicate that aptamers would be applicable as a useful and cost-effective tool to identify the presence of the particular target protein with their specific protein partners.

1. Introduction

Aptamers are single-stranded oligonucleotides that form stable three-dimensional structures capable of binding with high affinity and specificity to a variety of molecular targets. Aptamers against a specific target are generated using an iterative approach called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [1]. Aptamers have advantages over more antibodies in that they are poorly immunogenic, stable, and often bind to a target molecule more strongly than do antibodies. Furthermore, producing an aptamer is more cost-advantageous than an antibody because it can be synthesized easily and in large quantities by in vitro transcription, PCR, or chemical synthesis [2,3]. Aptamers have been shown to be useful as therapeutic agents [4], diagnostic tools [4], biochemical detection [5], and affinity-purification [6,7].

Antibodies are widely used as a tool in protein identification and quantification methods. Immunoprecipitation (IP) is one of the most widely used methods for antigen detection and purification. An important characteristic of IP reactions is their potential to deliver not only the target protein, but also other cellular proteins that interact with the target. IP of intact protein complexes (i.e. antigen along with any proteins or ligands that are bound to it) is known as co-immunoprecipitation (Co-IP). Co-IP is a popular technique to identify physiologically relevant protein–protein interactions by using target protein-specific antibodies. However, most commonly encountered problems with IP and Co-IP approach is interference from antibody heavy and light chains in gel analysis. Co-precipitated antibody with the target can obscure the results. The ideal situation would be to conduct the Co-IP without contamination of the eluted antigen with antibody. Aptamer is an oligonucleotide that will not contribute to protein/peptide background that can interfere with subsequent analysis.

In this study, the aptamer-mediated cellular protein precipitation which is a technique to identify physiologically relevant protein–protein interactions by using target protein-specific aptamers is provided. This assay is performed by using both aptamer–conjugated magnetic agarose beads and biotinylated aptamer with streptavidin-coated magnetic beads, and confirmed its superior performance over antibody based methods.

2. Materials and methods

2.1. Modified systematic evolution of ligands by exponential enrichment (SELEX)

The advanced SELEX technology was used as described by Gold et al. [1]. Briefly, aptamers were selected from a DNA library containing a 40-nucleotide randomized region in which 5-(*N*-benzylcarboxyamide)-2′-deoxyuridine (Bz-dU) or 5-(*N*-naphthylcarboxyamide)-2′-deoxyuridine (Nap-dU) was substituted for dT. The oligonucleotides contained a central randomized region of 40

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nucleotides flanked by two conserved flanking regions of 17 nucleotides (5′-GAGTGACCGTCTGCCTG-40N-CAGCCACACCACCAGCC-3′). Twenty-five thermal cycles were conducted at 93 °C for 30 s, 52 °C for 20 s, and 72 °C for 60 s. The SELEX process was performed at 37 °C. A mixture of 1 mmol of aptamer library dissolved in a buffer solution (40 mM HEPES/pH 7.5, 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 0.002% Tween 20) was heated at 95 °C for about 3 min, and then slowly cooled to 37 °C at 0.1 °C/s for re-folding. Aptamer library was pre-incubated with His tag magnetic bead (Invitrogen, Grand Island, NY) to eliminate non-specific binder to magnetic bead. Aptamer library in supernatant were incubated with purified 10 pmol of target proteins for 30 min and then target proteins captured by contacting with His tagged beads for 10 min. Aptamers bound to the target proteins were eluted with 2 mM NaOH solution and amplified via PCR reaction. The resulting aptamers were used in the next SELEX round.

2.2. Cloning and sequencing of selected aptamers

After 8 rounds of SELEX, eluted aptamers were amplified by QPCR using primers, and then cloned into TA cloning Kit (Solgent, Korea). Fifty colonies were picked for each sample and the cloned parts were sequenced by Solgent. Sequences were aligned using the 'aptamer motif searcher', an in-house program of Aptamer Sciences Inc., and a pattern analysis was performed.

2.3. Binding affinity assays

The aptamer–protein equilibrium dissociation constants (K_d) were determined by the nitrocellulose-filter binding method [8]. For all binding assays, aptamers were dephosphorylated using alkaline phosphatase (New England Biolab, Beverly, MA), 5-end labeled using T4 polynucleotide kinase (New England Biolabs) and [^{32}P]-ATP (Amersham Pharmacia Biotech, Piscataway, NJ) [9]. Direct binding assays were carried out by incubating ^{32}P-labeled aptamer at a concentration of less than 10 pM and protein at concentrations ranging from 1 mM to 10 fM in selection buffer at 37 °C. The fraction of bound aptamer was quantified with a PhosphorImager (Fuji FLA-5100 Image Analyzer, Tokyo, Japan). Raw binding data were corrected for nonspecific background binding of radiolabeled aptamer to the nitrocellulose filter.

2.4. Cell culture

The human skin carcinoma cell line (A431) and Rat-1 cells stably expressing the human insulin receptor (Rat-1/INSR) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 10 U/mL penicillin and 10 μg/mL streptomycin (Gibco) in a 5% CO_2-humidified chamber at 37 °C.

2.5. Conjugation of magnetic agarose bead with aptamers

Antibodies were immobilized to Magnetic beads (Dynabeads M270 amine) (#143-07D, Invitrogen) according to the manufacturer's instructions. Thiol aptamers (SH-aptamers) were conjugated to amino magnetic agarose beads (BioScience Beads Division, RI). Pack 0.5 ml magnetic beads in column or centrifuge tube and wash thoroughly with activation buffer (1 M NaCl, 0.05 M $NaHPO_4$, 1 mM EDTA pH 7.4). The beads were prepared 50% suspension in the column with the activation buffer and added iodoacetic NHS (FW 283; Sigma/Aldrich #I9760) to 15 μmol/ml packed gel. Beads were incubated with shaking for 2 h at ambient temperature. Acetic anhydride was added in a final concentration of 0.05 M in the 50% suspension. Beads were washed with coupling buffer (1 M NaCl, 0.05 M-Bicarb-NaOH, 1 mM EDTA pH 9.0). The activated thiol aptamer was added directly to the activated magnetic beads under N_2 and allowed to couple overnight.

2.6. Aptamer-mediated cellular protein precipitation (aptoprecipitation, AP) assay

About 4×10^6 cells (70–80% confluence) in 100 mm culture dish were solubilized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1% NP-40) containing protease inhibitor cocktail (Roche), and the cell lysates were incubated on ice for 10 min. The cell lysates were clarified by centrifugation at 12,000*g* for 10 min at 4 °C after brief sonication. The cleared lysates were mixed with either biotinylated aptamer or aptamer–magnetic agarose beads, 200 μg/ml salmon sperm DNA (ssDNA) (Ambion), and final 1 mM dextran sulfate ($DxSO_4$, Framingham, MA, USA). After incubating for 2 h or overnight at 4 °C, the mixed solution washed four times with detergent-free wash buffer.

2.7. Aptamer-mediated cellular protein co-precipitation (Co-aptoprecipitation, Co-AP) assay

Cells were starved to serum-free medium overnight (16–18 h) and were stimulated with 100 nM insulin (Sigma–Aldrich, ON, Canada) or EGF (Sigma–Aldrich) for 5 min at 37 °C. Cell monolayers were washed twice with phosphate buffered saline (PBS) and lysed into lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1% NP-40) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche) by incubation for 30 min on ice. The cell lysates were clarified by centrifugation at 12,000*g* for 10 min at 4 °C. The cleared lysates were mixed with aptamer–magnetic agarose beads, 200 μg/ml ssDNA, and final 0.01 mM dextran sulfate. After incubating overnight at 4 °C, the mixed solution washed four times with detergent-free lysis buffer.

2.8. Elution of target proteins

The bound proteins were eluted by 20 mM Triethylamine (TEA), pH 11.3 for 15 min at 25 °C with gentle mixing. Place the tube on a magnetic stand and transfer the supernatant to new tube and add 3 μl of 2 M Tris–HCl (pH 7.0) to the supernatant for the neutralization and mix immediately.

2.9. SDS–PAGE and Western blot

Total cell lysates or eluate were boiled in a loading buffer and subjected to SDS–PAGE (4–15% gradient gel) and stained by SYPRO ruby proteins (Invitrogen). Stained images were visualized using a Fuji FLA-5100 Image Analyzer. For Western blot analysis, samples were subjected to SDS–PAGE and blotted onto nitrocellulose membranes. After blocking in 5% skim milk in TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), membranes were probed with specific antibodies and proteins were visualized with peroxidase-coupled secondary antibodies using the ECL system (Amersham Biosciences).

3. Results and discussion

3.1. Generation of anti-EGFR and INSR aptamers with high affinities via advanced SELEX using a modified nucleoside

In order to generate DNA aptamers against EGFR (ErbB-1) or INSR, we used a truncated each protein containing the extracellular domain that was expressed in mouse myeloma cell line. For INSR-binding aptamers, a His-tagged fusion of the extracellular domain (28–750 amino acids (α subunit) and 751–944 with a C-terminal