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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

10-his tag (β subunit)) (R&D Systems, #1544-IR/CF) was partitioned on Talon (Dynal, Lake Success, NY) beads. For EGFR-binding aptamers, an Fc fusion of the extracellular domain (100–330 amino acids) (R&D Systems, #344-ER) was biotinylated and partitioned on streptavidin MyOne-SA (Dynal) beads. Advanced SELEX, using Bz-dU or Nap-dU instead of Thymidine, was performed to acquire aptamers with high affinity and specificity [1,9,10].

The aptamers against the EGFR and INSR were obtained from a pool of 1×10^{14} DNAs that consisted of a 40-nt randomized region with 17 and 17 primer sequences for PCR amplification. The DNA pool was mixed with the target proteins and then the target proteins–DNA complex was captured on a nitrocellulose filter. Selections were carried out with gradual reductions in the protein quantity and the DNA amounts in each round. After 8 rounds of selection, four aptamers (2369-1-1, -11-1, -12-1, -27-1) with high affinities to the purified EGFR proteins were obtained (Table 1), and three aptamers (1652-17, -18, -36) with high affinities to the purified INSR proteins were obtained (Table 2). The affinity values of aptamers ranged K_d = 0.6–12 nM. As a result of a secondary structure prediction study of the 2369-27-1 and 1652-17 clones with a web-based program Mfold, a minimized 2369-27-2 and 1652-49 were successfully obtained without any loss of the binding ability to the EGFR and INSR, respectively (Tables 1 and 2). These 2369-27-2 and 1652-49 clones were used for the following AP and Co-AP assay and the sequence data for these clones are presented in Table 3. Aptamers were synthesized on an automated solid phase DNA synthesizer. A 0.1 M solution of phosphoramidite in anhydrous acetonitrile was used for the synthesis. All of the oligonucleotides were characterized by LC ESI-MS and the purity was assessed by HPLC and capillary electrophoresis.

3.2. Reduction of nonspecific proteins using polyanionic competitor

To reduce background nonspecific proteins encountered in aptamer-mediated cellular protein precipitation, the AP experiment using EGFR aptamer (2369-27-2) was conducted using dextran sulfate as a competitor. Different concentrations of dextran sulfate ranging from 0.01 to 1.0 mM were used to block nonspecific proteins in cell lysates (Fig. 1). EGFR bands were detected and nonspecific protein bands were reduced at an increased concentration of dextran sulfate. This result indicates that dextran sulfate can reduce nonspecific proteins in AP assays, thereby allowing highly specific interaction between the aptamers and the EGFR. We reasoned that a polyanionic competitor would substantially reduce nonspecific binding. Modified aptamers contain novel base modifications (Bz-dU or Nap-dU) that mediate hydrophobic interactions at the protein–aptamer interface, thereby achieving high affinity binding. Modified aptamers are selected using a kinetic challenge with dextran sulfate, thus favoring slow target dissociation rates and minimizing ionic contributions to binding. Our results are in good agreement with the result work of Shashi et al. [11] reported that dextran sulfate is useful for reducing background of nonspecific proteins in immunohistochemistry.

3.3. Performance of biotinylated aptamer in protein precipitation assay

In order to compare the performance of aptamers and antibodies in cellular protein precipitation assays, A431 cell lysates that express the human EGFR were incubated with biotinylated EGFR aptamers and dextran sulfate, followed by incubation with streptavidin magnetic beads as described in Section 2. As shown in Fig. 2A, EGFR proteins were precipitated from A431 cell extract using the biotinylated EGFR aptamer. An intense EGFR band was clearly obtained by using the biotinylated EGFR aptamer, while a relatively weak EGFR band was obtained when precipitating with anti-EGFR antibody. An intense EGFR band was also obtained with high-pH elution buffer (20 mM TEA, pH 11.3).

Protein precipitation assays were also performed with Rat-1/INSR cell overexpressing insulin receptors. Briefly, Rat-1/INSR cell lysates that express the human INSR were incubated with biotinylated INSR aptamers (1652-49) and dextran sulfate, followed by incubation with streptavidin magnetic beads as described in Section 2. Fig. 2B shows that the INSR proteins were precipitated from Rat-1/INSR cell extract using the biotinylated INSR aptamer. An intense

Table 2
Binding affinities (K_d values) of aptamers against INSR proteins.

INSR aptamers	1652-17	1652-18	1652-36	1652-49
K_d (nM)	2.8	10	7.9	3.3

Table 3
Sequences of the EGFR and INSR binding aptamers used for AP and Co-AP assay. '5' indicates the 5-Bz-dU-modified oligonucleotide and '6' indicates the 5-Nap-dU-modified oligonucleotide of aptamers.

Clone	Sequence
2369-27-2	AGTTCAGCCCCGG66A6ACGG6C6CA6GCC6G6GCG66 6AACC6AGACCA
1652-49	GCCTG5AAGG555AAGC55GGCC5AA5GG5GC5A5CAGGC5C

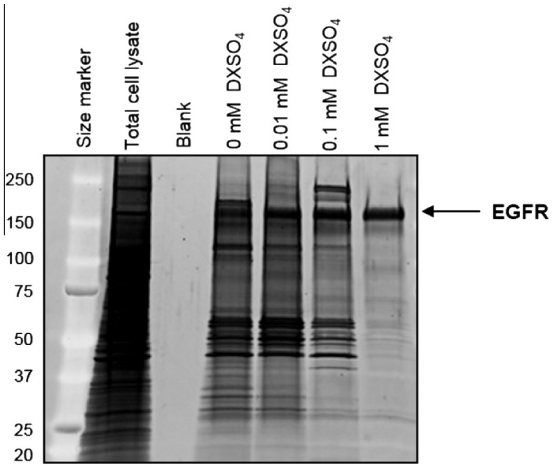

Fig. 1. Reduction of nonspecific proteins using dextran sulfate. A431 cell lysates (1 mg/lane) were incubated with biotinylated EGFR aptamers (2369-27-2) (20 pmol) and dextran sulfate. The mixed solution was further incubated with streptavidin magnetic beads. Gels were stained with SYPRO Ruby. Lane 1 indicates size marker. Lane 2 indicates A431 cell lysates corresponding to 20 μg of total cellular proteins. Lane 3 indicates Blank lane. Lanes 4–7 indicate eluate by boiling SDS loading buffer after AP with different concentration of dextran sulfate ranging from 0 to 1.0 mM.

Table 1
Binding affinities (K_d values) of aptamers against EGFR proteins.

EGFR aptamers	2369-1-1	2369-11-1	2369-12-1	2369-27-1	2369-27-2
K_d (nM)	11.7	6.3	7.1	0.62	0.99

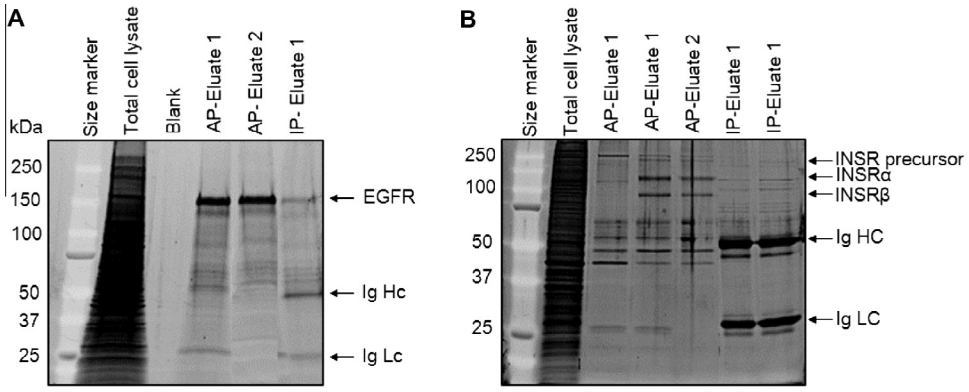

Fig. 2. Aptoprecipitation of target proteins with biotinylated aptamers. AP of EGFR protein with biotinylated EGFR aptamer (2369-27-2) (A). A431 cell lysates (1 mg/lane) were incubated with either biotinylated EGFR aptamers (20 pmol) or anti-EGFR antibody (20 pmol). The mixed solution was further incubated with either streptavidin magnetic beads for aptamer or Protein-A bead for antibody. AP of INSR protein with biotinylated INSR aptamer (1652-49) (B). Rat-1/INSR cell lysates (1 mg/lane) were incubated with either biotinylated INSR aptamers (40 pmol) or anti-INSR antibody (40 pmol). The mixed solution was further incubated with either streptavidin magnetic beads for aptamer or Protein-A bead for antibody. The bound protein was eluted in either SDS-sample buffer (eluate 1) or high-pH elution buffer (20 mM TEA, pH 11.3) (eluate 2) and separated by SDS–PAGE (4–15% gradient gel). The gel was directly stained with SYPRO ruby. TCL: total cell lysate.

INSR bands (INSRα and INSRβ) were clearly obtained by using the biotinylated INSR aptamer, while a relatively weak INSR band was detected when precipitating with anti-INSR antibody. An intense INSR bands (INSRα and INSRβ) were also obtained by high-pH elution buffer. Both INSRα and INSRβ proteins were precipitated from Rat-1/INSR cells but not from Rat-1 cells when precipitating with either INSR aptamer or antibody. These results indicate that the biotinylated aptamers efficiently precipitate target proteins from a complex protein mix, while antibodies precipitate extraordinarily small amounts of target proteins and the performance of aptamers in terms of specificity and selectivity is significantly superior compared to antibodies in protein precipitation assays.

3.4. Performance of aptamer-magnetic agarose beads in protein precipitation assay

To compare the performance of aptamers and antibodies conjugated magnetic bead, A431 cell lysates that express the human EGFR were incubated with either EGFR aptamer (2369-27-2)-magnetic agarose beads or EGFR antibody-magnetic beads as described in Section 2. As shown in Fig. 3A, it should be noted that many proteins which are not related with EGFR were detected in IP with antibody from bead-bound proteins (lanes 6–7) and the proteins eluted from the bead (lane 8). Moreover, EGFR protein was not clearly detected from IP with antibody. On the other hand, much less non-specific protein-binding was observed in AP with aptamer from bead-bound proteins (lane 3) and the proteins eluted from the bead (lane 4) without sacrificing specific binding of EGFR. EGFR aptamers were highly specific to EGFR, while anti-EGFR antibody precipitated extraordinarily small amounts of EGFR.

Protein precipitation assays were performed with another Rat-1/INSR cell expressing insulin receptors. Rat-1/INSR cell lysates that express the human INSR were incubated with either INSR aptamer (1652–49)-magnetic agarose beads and INSR antibody-magnetic beads as described in Section 2. As shown in Fig. 3B (upper data), it should be noted that many proteins which are not related

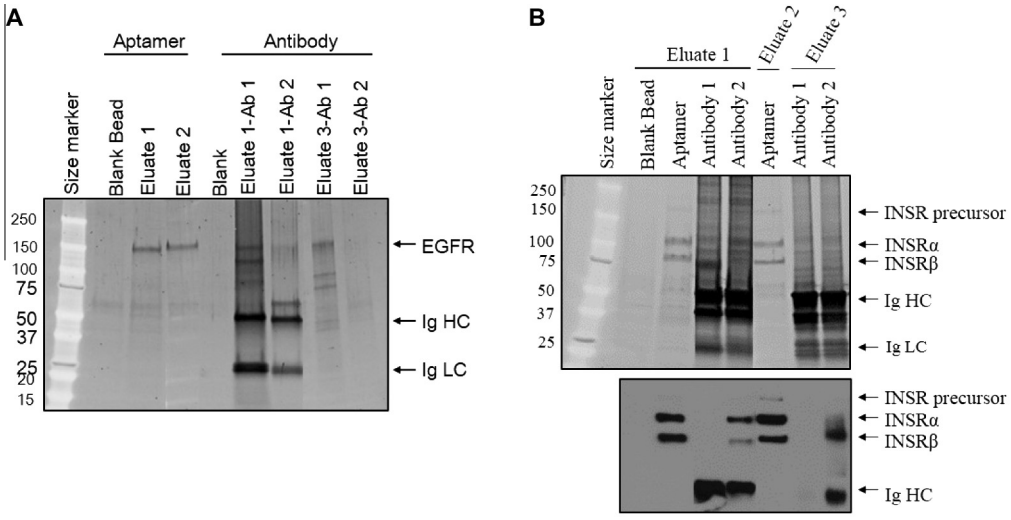

Fig. 3. Aptoprecipitation of target proteins with aptamer–magnetic agarose beads. AP of EGFR protein with EGFR aptamer (2369-27-2)–magnetic agarose beads (A). A431 cell lysates (1 mg/lane) were incubated with either EGFR aptamer (50 pmol)–magnetic agarose beads or anti-EGFR antibody (50 pmol)-magnetic beads (Dynabead M270). The bound protein was eluted in either SDS-sample buffer (eluate 1), high-pH elution buffer (20 mM TEA, pH 11.3) (eluate 2) or low-pH elution buffer (pH 4.0) (eluate 3). AP of INSR protein with INSR aptamer (1652-49)–magnetic agarose beads (B). Rat-1/INSR cells lysates (1 mg/lane) were incubated with either INSR aptamer (50 pmol)–magnetic agarose bead or anti-INSR antibodies (50 pmol)-magnetic bead (Dynabead M270). The bound protein was eluted in either SDS-sample buffer (eluate 1), high-pH elution buffer (eluate 2, aptamer) or low-pH elution buffer (eluate 3, antibodies). Proteins were separated by SDS–PAGE (4–15% gradient gel). The gel was directly stained with SYPRO ruby (upper image). Proteins were also blotted onto a PVDF membrane and Western blot was probed with specific antibodies (lower image). Blank bead: aptamer (reverse complement sequence of EGFR or INSR aptamer)–coupled magnetic agarose beads is used as a control.

with INSR were detected in IP with antibody from bead-bound proteins (lanes 4–5) and the protein eluted from the bead (lanes 7–8). On the other hand, much less non-specific protein-binding was observed in AP with aptamer from bead-bound proteins (lane 3) and the proteins eluted from the bead (lane 6) without sacrificing specific binding of INSR-α and INSR-β. INSR aptamers were highly specific to INSR, while anti-INSR antibody precipitated extraordinarily small amounts of EGFR. To identify aptoprecipitated target proteins, Western blot analysis was performed (Fig. 3B, below data). As a result, all INSRα, INSRβ, and INSR precursor were identified in AP with aptamer under both boiled and eluted samples, while any of INSR proteins were not detected in IP with antibody (Santa cruz anti-Irβ). However, all INSRα, INSRβ, and INSR precursor were detected by anti-IRα (Invitrogen) under both boiled and eluted samples. These results indicated that aptamers demonstrate less interference and higher specificity than antibodies in protein precipitation assays.

3.5. Identification of physiologically relevant protein–protein interactions using aptamers

To verify the performance of EGFR aptamers in Co-AP assay, A431 cells were starved to serum-free medium overnight (16–18 h) and were stimulated with 100 nM EGF (Sigma–Aldrich). A431 cell lysates were incubated with EGFR aptamer (2369-27-2)-magnetic agarose beads and dextran sulfate as described in Section 2. As shown in Fig. 4A, EGFR interacting proteins such as PLC-γ1, Shc, PI3K, Akt2, and Grb2 were identified in aptamer-mediated Co-AP assay, while none of EGFR interacting proteins was detected with control aptamer–magnetic agarose beads. The PLC-γ1, Shc, PI3K, Akt2, and Grb2 proteins have been implicated in EGFR signaling. The binding of EGF at the cell surface induces dimerization of EGFR, which results in the activation of EGFR tyrosine kinase activity and *trans*-autophosphorylation. Sites of tyrosine autophosphorylation in an activated EGFR bind signaling proteins that contain phosphotyrosine (pTyr)-binding domains such as Src homology 2 and phosphotyrosine binding [12,13]. These signaling proteins include Grb2, Shc, PLC-γ1, PI3K and Akt2 [14,15].

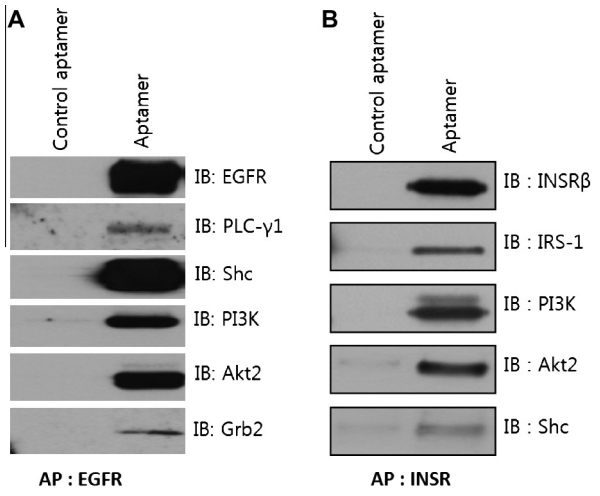

Fig. 4. Co-aptoprecipitation of target proteins and their interacting proteins with aptamer–magnetic agarose beads. Co-AP of EGFR and their interacting proteins from A431 cell lysate using aptamer–magnetic agarose bead (A). Subconfluent A431 cell culture was starved overnight and stimulated with 100 nM EGF. A431 cell lysates (1 mg/lane) were incubated with either EGFR aptamer (200 pmol)–magnetic agarose beads or control EGFR aptamer (200 pmol)–magnetic agarose beads. The bound protein was eluted and separated with SDS–PAGE and blotted onto a PVDF membrane. The membrane was probed with specific antibodies [anti-EGFR Ab (Cell signaling #2232), anti-PLC-γ1 Ab (R&D systems #MAB3288), anti-Shc Abs (Cell signaling #2432), anti-PI3K Ab (Santa cruz #sc-1637), anti-Akt Ab (Cell signaling #4691), and anti-Grb2 Abs (R&D systems #MAB3846)]. Co-AP of INSR and their interacting proteins from Rat-1/INSR cell lysate using aptamer–magnetic agarose beads (B). Subconfluent Rat-1/INSR cell culture was starved overnight and stimulated with 100 nM insulin. Rat-1/INSR cell lysates (1 mg/lane) were incubated with either INSR aptamer (200 pmol)–magnetic agarose bead or control EGFR aptamer (200 pmol)–magnetic agarose beads. The samples were separated by SDS–PAGE and blotted onto a PVDF membrane. The membrane was probed with specific antibodies [anti-IR β Ab (Cell signaling #3025), anti-IRS1 Ab (Millipore #06-248), anti-Akt Ab (Cell signaling #4691), anti-PI3K Ab (Santa cruz #sc-1637), anti-Shc Abs (Cell signaling #2432)]. TCL: total cell lysate. Control aptamer: reverse complement sequence of EGFR or INSR aptamer–coupled magnetic agarose beads is used as a control.

To verify the performance of INSR aptamers, Co-AP was performed with another Rat-1/INSR cell expressing insulin receptors. Rat-1/INSR cells were starved to serum-free medium overnight (16–18 h) and were stimulated with 100 nM insulin (Sigma–Aldrich). Rat-1/INSR cell lysates were incubated with INSR aptamer (1652-49)-magnetic agarose beads and dextran sulfate as described in Section 2. As a result, INSRβ was clearly detected in Co-AP eluted proteins from bead-bound proteins, while INSRβ protein was not detected with control aptamer–magnetic agarose beads (Fig. 4B). INSR interacting proteins such as IRS-1, PI3K, Akt2, and Shc were identified in aptamer-mediated Co-AP assay, while any of INSR interacting proteins were not clearly detected with control aptamer–magnetic agarose beads.

The IRS-1, Shc, PI3K, and Akt2 proteins have been implicated in insulin receptor (INSR) signaling. Identification of the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway and activating receptor tyrosine kinases (RTKs) began in earnest in the early 1980s through vigorous attempts to characterize INSR [16]. These led to the identification of the components and mechanism of INSR signaling via INSR substrate (IRS) proteins to PI3K and consequent PKB/Akt-mediated activation by 3-phosphoinositide-dependent protein kinase 1 (PDK1). The Shc proteins have also been implicated in INSR signaling. It was demonstrated by using sensitive two-hybrid assay of protein–protein interaction that SHC interacts directly with the INSR. SHC interacts directly with the INSR and that phosphorylation of Tyr-960 within the INSR juxtamembrane domain is necessary for efficient interaction [17].

We succeeded in generating DNA aptamers that target EGFR and INSR proteins in cells with high affinity and specificity. Furthermore, we developed a reproducible aptamer-mediated protein precipitation technique that is a novel sample preparation tool used for concentration, and isolation of a particular protein from complex biological samples and is applicable to identify physiologically relevant protein–protein interactions. AP and Co-AP experiments indicated that the binding affinities of EGFR and INSR aptamers were remarkably higher than those of anti-EGFR and INSR antibodies (Figs. 2 and 3).

Since aptamer is an oligonucleotide that will not contribute to protein background that can interfere with subsequent analysis, we will evaluate to detect the proteins specific to the EGFR including many known and (potentially) novel interactors as well as some EGFR posttranslational modifications (PTMs).

Our aptamers showed significant and specific binding to their target proteins in aptamer-mediated AP and Co-AP assays that can be a useful tool for the studies of physiologically relevant protein–protein interactions. These aptamers have potential use in the quantitative analysis of the amount of target proteins with the double-aptamer sandwich detection. These aptamers have another potential use in the cell separation assays. Efficient selection and enumeration of low-abundance biological cells are highly important in a variety of applications. Aptamer-mediated cell separation technology may provide the specificity of monoclonal aptamers for effortless separation of highly purified cells that are immediately ready for downstream applications. Cells are captured by magnetic

particles using aptamer, and then easily separated from unwanted cells with a magnet. Furthermore aptamers bind to target proteins with native conformational structure, while antibodies are difficult, so such approaches will gives big advantages for low-abundance-cell isolation and enumeration.

Acknowledgments

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