Aptameric Probe Specifically Binding Protein Heterodimer Rather Than Monomers

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Dimerization of proteins occurs frequently and plays integral roles in biological processes. However, no single molecular probe is available for in situ detection of protein dimers on cells and tissues because of the difficulty of isolating complete protein dimers for probe preparation and screening, which has greatly hampered the biomedical study of protein dimers. Herein, a G-rich DNA aptamer (termed BG2) that only binds alkaline phosphatase (AP) heterodimers rather than monomers is reported. This aptamer is generated by the cell-SELEX (systematic evolution of ligands by exponential enrichment) technique and proves to fold into a duplex stabilized antiparallel G-quadruplex structure. Using BG2 as molecular probe, AP heterodimers are found to be expressed on several kinds of cancer cells. As an affinity ligand, BG2 could isolate AP heterodimers from cell lysate. BG2 is also demonstrated to be applicable for tumor imaging in mice xenografted with cells highly expressing AP heterodimers. AP isozymes are found in several tissues and blood throughout the body, but the function and tissue distribution of AP heterodimers are totally unknown; therefore, BG2 could serve as a molecular probe to uncover the mystery of AP heterodimers. The generation of aptameric probes by cell-SELEX will open up a new situation for the study of protein dimers.

1. Introduction

Dimerization of proteins occurs frequently in cells and plays important roles in various biological processes and cancer development.[3–5] However, because most of the protein dimers are assembled by noncovalent protein–protein interactions, they are usually disassembled during cell lysis and protein isolation, making the detection and identification of protein dimers very difficult. Although some experimental techniques have been developed for isolation and identification of protein dimers, such as blue-native polyacrylamide gel electrophoresis (PAGE), co-immunoprecipitation, protein-fragment complementation assays, mass spectrum, and X-ray crystallography,[6] the in situ detection of protein dimers on cells and tissues is still a serious challenge because of the lack of molecular probes for directly recognizing a protein dimer. For example, alkaline phosphatase (AP) homodimers and heterodimers formed by intestinal AP (IAP) and placental AP (PLAP) are reported several decades ago,[7–9] but the distributions and functions of AP dimers on human cells and tissues are absolutely unknown due to lack of efficient probes for the direct recognition of AP dimers. Recently, great efforts have been made to visualize protein dimers on cells, resulting in the development of methods such as real-time tracking receptor dimerization on live cells by single-molecule fluorescence microscopy based on Förster resonance energy transfer between fluorescent protein-fused receptors,[10] imaging protein dimerization through proximity ligation, and proximity-induced DNA assembly of two protein-specific antibodies or aptamers.[11] However, there is no method that could be used for in vivo or high throughput detection of protein dimers. To our knowledge, no single molecular probes (antibodies and aptamers) have been reported to recognize a protein dimer (heterodimer and homodimer) rather than its monomers.

For single probe that can discriminate a protein dimer from its monomers, the probe should bind at a site formed by two monomers. Typically, antibodies are large Y-shaped proteins (=150 kDa), and they recognize a particular epitope on antigens with about 4–12 amino acids in size via the Fab’s variable region (two tips of the “Y”) (Scheme 1a).[12] Therefore, antibodies are hard to bind an epitope spanning across a dimeric protein. In addition, antibodies are usually obtained by immunizing the animals with antigens. However, it is hard to obtain pure protein dimers to generate antibodies. Aptamers are synthetic single-stranded oligonucleotides with high selectivity and specificity toward their targets.[13] Compared with antibodies,
aptamers are much smaller in size (≈6–30 kDa),[10] which either bind a small epitope on target proteins[11] (Scheme 1b) or fit in a binding pocket of large proteins or protein complexes. Therefore, aptamers may access to the pocket formed by a dimeric protein and interact with the adjacent amino acid residues of two monomers (Scheme 1c). Aptamers are generated by an iterative in vitro selection process known as systematic evolution of ligands by exponential enrichment (SELEX) against various targets, such as pure proteins, cells, and tissues.[9] Selection using live cells as target (cell-SELEX) is able to generate aptamers that recognize prior unknown molecular signatures on cell surface.[12] Because proteins on live cells are present in their native and active states, cell-SELEX has the potential to obtain aptamers that recognize unknown protein dimers or protein complexes.

Herein, we report an aptamer generated by cell-SELEX technique that specifically bind alkaline phosphatase heterodimers rather than monomers. The applications of this aptamer for the detection and isolation of AP heterodimers were performed.

2. Results

2.1. Aptamer Generation and Characterization

The aptamer was selected from a restricted library by using HeLa cells as target cells. The restricted library was favorable to form a duplex stabilized G-quadruplex structure instead of a totally random library, which contained 5 G-tracts in the central regions to favor the formation of G-quadruplex, a YRRRRR and a YYYYYR sequence (Y = C or T; R = A or G) within the random region next to the primer binding regions to increase the probability of forming the duplex (see the Supporting Information for details). The cell-SELEX was performed as previously described (see the Experimental Section and Figure S1 in the Supporting Information).[13] After five rounds of selection, the enriched DNA pool of the fifth round was sequenced by high throughput sequencing. About 15 000 sequences were obtained, and 42% of them contained a conserved G-rich motif, GGGTCGGTGTTGGTGTATGGGG, suggesting that it may be the binding motif of aptamers. The most abundant sequence, BG2 (Figure 1a; Table S1, Supporting Information, not including the primer sequences), was chosen for further characterization.

The secondary structure prediction showed that BG2 adopted a hairpin structure with a G-rich loop (Figure 1a). The binding assays showed that BG2 strongly bound to MCF-7, HeLa, LoVo, HepG2, and SMMC-7721 cell lines, whereas very weakly bound to HCT-116, and nonbinding to PC-3, Jurkat-6E, SH-SY5Y, and HEK293 cell lines (Figure 1b). Since BG2 showed the strongest binding to LoVo cell line, this cell line was mainly used for further study. The apparent equilibrium dissociation constant (K_d) of BG2 was measured to be 2.5 ± 0.3 × 10^{-9} M (Figure 1c) by flow cytometry. BG2 could keep similar binding ability to LoVo cells at 4, 25, and 37 °C, even in the culture medium containing 10% FBS. In addition, BG2 also bound to fixed cells (Figure S2, Supporting Information).

Figure 1. Characterization of aptamer BG2. a) Proposed secondary structure of aptamer BG2. b) The selectivity assay of BG2 to different cell lines. c) Binding curve of BG2 to LoVo cells determined by flow cytometry. d) CD spectra of BG2, BG4, and BG2t.
In order to reveal the binding structure of BG2, a mutation assay was performed (Table S1, Supporting Information). Replacing the stem of BG2 with a new stem (BG4) caused its $K_d$ increase to $11.5 \pm 2.3 \times 10^{-9}$ M (Figure S3a, Supporting Information). Furthermore, removing the stem of BG2 (BG2c) caused its $K_d$ increase to $9.3 \pm 1.6 \times 10^{-9}$ M (Figure S3b, Supporting Information). Further removing the G-tract at the 3′-end and 5′-end of BG2c respectively (BG2c2 and BG2c3) caused the loss of their binding ability to LoVo cells (Figure S3c, Supporting Information). Replacing the G-tracts in the loop of BG2 with T-tract (BG2t) or mutating G16 to A (BG2a) resulted in the loss of their binding ability. Deleting the TATGAT in the loop (BG2m) also caused the loss of its binding ability. These mutation assay results suggest that the G-rich loop is the binding motif of BG2, the stem only stabilizes the binding structure, and the G-tracts and the TATGAT sequences in the G-rich loop are essential for aptamer binding.

To illuminate whether the G-rich loop folds into G-quadruplex, a circular dichroism (CD) spectral analysis was carried out (Figure 1d). The strong binding sequences, BG2 and BG4, showed a positive band around 295 nm and a negative band at 260 nm, indicating the formation of an antiparallel G-quadruplex. The non-G-tract sequence, BG2t, did not show any CD signals of G-quadruplex. The nonstem sequences, BG2c, BG2c2, and BG2c3, did not show clear CD signals of G-quadruplex (Figure S3d, Supporting Information), suggesting that these sequences could not form stable and unique G-quadruplexes without the help of stem. BG2m also showed the CD signals of antiparallel G-quadruplex, but it did not bind to LoVo cells, suggesting that the removed sequence, TATGAT, may involve in the binding to target cells.

2.2. Target Identification of Aptamer BG2

The target protein of BG2 was identified by stable isotope labeling with amino acids in cell culture (SI-LAC)-based quantitative proteomic analysis according to the protocol previously reported. After crosslinking of biotin-labeled BG2 or control sequence BC1 to LoVo cells by 1% formaldehyde, the candidate proteins were captured from cell lysate by streptavidin-coated sepharose beads, then digested with trypsin, and identified by LC-MS/MS analysis. Totally 114 valid proteins were identified (Table S2, Supporting Information). According to the abundance ratio of proteins captured by aptamer BG2 and control sequence BC1, five membrane proteins with the ratio higher than 20 were speculated as the candidate protein targets, i.e., intestinal-type AP, placental-type AP, germ cell AP (GCAP), neural cell adhesion molecule L1 (L1CAM), and HLA class I histocompatibility antigen (HLA-A). The MS quantification results for representative peptides of these proteins are shown in Figures S4–S8 in the Supporting Information.

Since three APs were identified with high SILAC ratio, and IAP and PLAP showed the highest score and sequence coverage in the candidate proteins, APs, especially IAP and PLAP, are the most possible targets of BG2. Furthermore, according to the information in ProteomicsDB (https://www.proteomicsdb.org), HLA-A expressed in Jurkat and PC-3 cell lines was higher than in HepG2 and MCF-7 cell lines; however, our results showed that BG2 bound HepG2 and MCF-7 cells but did not bind Jurkat and PC-3 cells, suggesting that HLA-A may not be the direct target of BG2. L1CAM was found highly expressed on surface of SH-SYSY cell line (Figure S9a, Supporting Information), but BG2 could not bind SH-SYSY at all (Figure 1b). When knocked down the expression of L1CAM on LoVo cells using small interfering RNA (siRNA), the BG2 binding was not affected (Figure S9b, Supporting Information), suggesting that L1CAM is not the direct target of BG2 either. Several reports have documented that IAP and PLAP can bind immunoglobulin; both L1CAM and HLA-A are immunoglobulin-like proteins, thus they might be isolated together with IAP and PLAP by BG2.

2.3. Discovery of AP Heterodimers as Molecular Target of BG2

To confirm that APs are the target of BG2, we performed an aptamer pull-down experiment and tested the pull-down proteins using Alkaline Phosphatase Assay Kit. The BG2 pull-down proteins showed very high AP activity (Figure 2a), confirming that the molecular target is at least one isozyme of APs.

In order to figure out which AP is the real target of BG2, western blot assay and immunoassay by flow cytometry and confocal imaging were performed. It should be noticed that in the western blot assay, the commercially available anti-IAP monoclonal antibodies (see the Supporting Information) showed strong cross-reaction to PLAP because of the 85% homology between IAP and PLAP. The western blot assay showed that IAP and PLAP could be pulled down by aptamer BG2 but not by control sequences, BC1 (Figure 2b; Table S1, Supporting Information). The flow cytometry assay showed that both IAP and PLAP highly expressed on BG2-binding cells (LoVo, HeLa, and MCF-7), but lowly expressed on BG2-nonbinding cells (Jurkat and PC3) (Figure S10, Supporting Information). The confocal imaging and flow cytometry showed that LoVo cells could be costained by BG2 (labeled with Alexa Fluor 647) and anti-IAP/anti-PLAP antibodies, respectively (Figure 2c,d). These results suggest that BG2 might bind both APs, i.e., IAP and PLAP, because of their high homology.

However, we noticed some differences between the containing results of BG2/anti-IAP and BG2/anti-PLAP. In dot plots of flow cytometry, the fluorescence intensities of BG2 and anti-IAP on cells showed a near linear relationship (Pearson’s correlation coefficient = 0.703, $p < 0.01$), which suggests that BG2 and anti-IAP might bind to different epitopes of an IAP (Figure 2d). But the containing of BG2 and anti-PLAP did not show the near linear relationship (Pearson’s correlation coefficient = −0.016, $p > 0.05$). The competition experiment showed that the binding of anti-PLAP to LoVo cells was partly diminished by the addition of excess amount of unlabeled BG2 (Figure 2e). To figure out whether BG2 binds to PLAP, we conducted competition experiment using purified human PLAP (P3895, Sigma). PLAP did not compete the binding of BG2 to LoVo cells at all (Figure S11, Supporting Information). In order to mimic the real environment of PLAP on cell surface, PLAP protein was anchored on the membrane of Jurkat cells (BG2 nonbinding cells). As shown in Figure 2f, anti-PLAP bound the
PLAP anchored Jurkat cells, but BG2 could not. These results suggest that PLAP may not be the molecular target of BG2, which contradict the above results.

In order to figure out the real target of BG2, we knocked down the expression of endogenous IAP and PLAP on LoVo cells, respectively, using siRNAs (Figure 3a). Treatment of LoVo cells with siRNAs against IAP and PLAP, respectively, only reduced the binding of their corresponding antibody, and did not alter the binding of another antibody. However, treatment with siRNA against IAP, PLAP, or both of them significantly reduced the binding ability of BG2 to LoVo cells when compared with the treatment with control siRNAs. These results suggest that BG2 might not bind IAP or PLAP alone, but bind the heterodimer of them.

Therefore, we further transferred the plasmids of IAP, PLAP, or both of them to PC-3 cells (BG2-nonbinding cells). As shown in Figure 3b, BG2 did not bind the PC-3 cells over-expressed IAP or PLAP alone, but bound the PC-3 cells over-expressed both IAP and PLAP, suggesting that BG2 bound to the heterodimer of IAP and PLAP. Since GCAP was also identified with high SILAC ratio, we further tested whether GCAP bound to the heterodimer of IAP and PLAP. GCAP is highly homologous to PLAP with 98% amino acid similarity, and differ by only 10 amino acids,[18] no commercial available antibody can distinguish PLAP and GCAP; therefore, anti-PLAP was used to detect the expression of GCAP. As shown in Figure 3c, BG2 did not bind PC-3 cells transfected with only GCAP plasmid or both GCAP and PLAP plasmids, but bound to PC-3 cells transfected with IAP and GCAP plasmids together, suggesting that BG2 bound IAP–GCAP heterodimer, but did not bind PLAP–GCAP heterodimer. All the AP expression on cells was confirmed by western blot assay (Figure 3d).

When we re-examined the above results of flow cytometry (Figure S10, Supporting Information) and western blot (Figure 2b), we found that the expression of IAP on LoVo cells was much lower than that of PLAP. Interestingly, after being pulled down by BG2, the amount of IAP was comparable with PLAP (Figure 2b). The most likely explanation is that BG2 bound to the IAP–PLAP heterodimer, but not homodimers or monomers of them. In order to obtain the direct evidences of that BG2 bound IAP–PLAP heterodimer, we crosslinked proteins on surface of LoVo cells in situ by disuccinimidyl suberate (DSS), then pulled down proteins with BG2, and analyzed by western blot (the crosslinked proteins by DSS could not be broken under the used denaturation condition for SDS-PAGE). As shown in Figure 3e, without crosslinking, only IAP and PLAP monomers but no protein dimers were detected in cell lysate, the amount of IAP monomer was much lower than PLAP monomer (lane 1); after crosslinking, IAP, PLAP monomers, and PLAP homodimers were detected in cell lysate (lane 2); after crosslinking and BG2 pulling down, IAP and PLAP monomers and IAP–PLAP heterodimer were detected (no PLAP homodimer was detected), and the amount of IAP monomer was similar with PLAP monomer (lane 3). Because the efficiency of crosslinking was usually low, the uncrosslinked heterodimers pulled down by BG2 appeared in equal amount of IAP and PLAP monomers. This result provided the direct evidence that BG2 bound IAP–PLAP heterodimer.
2.4. Application of Aptamer BG2 as a Molecular Probe

Above results also demonstrated that aptamer BG2 could serve as a molecular probe for monitoring the AP heterodimers on cells, and as a ligand for purification. To further prove its feasibility for in vivo applications, tumor imaging in mice xenografted with LoVo (AP heterodimer positive cell) or PC-3 (AP heterodimer negative cell) cells was performed. As shown in Figure 4, after being intravenously injected with BG2-AF647, bright fluorescence was observed in tumor-implanted site of LoVo tumor-bearing mouse, while only very weak fluorescence was observed in tumor site of PC-3 tumor-bearing mouse. In contrast, there was almost no fluorescence detected in LoVo tumor-bearing mouse injected with L45-AF647 (control sequence). These results suggest that aptamer BG2 can only recognize tumor highly expressed AP heterodimer in vivo rather than AP monomers or homodimers normally expressed in other tissues. This is the first probe that could detect AP heterodimer in vivo.

Figure 3. Identification of AP heterodimers as molecular target of BG2. a) The binding of BG2, anti-IAP, and anti-PLAP to AP knock down LoVo cells, binding toward LoVo cells was assessed at 72 h after siRNA treatment. b,c) The binding of BG2, anti-IAP, and anti-PLAP to PLAP, GCAP, or IAP overexpressed PC-3 cells. d) Western blot assay of recombinant PLAP, GCAP, or IAP overexpressed on PC-3 cells. PC-3 cells were transfected with control plasmid (lane 1), IAP plasmid (lane 2), PLAP plasmid (lane 3), GCAP plasmid (lane 4), IAP + PLAP plasmids (lane 5), IAP + GCAP plasmids (lane 6), and PLAP + GCAP plasmids (lane 7). e) Western blot assay of AP dimers after in situ chemical crosslinking: lane 1, lysate of LoVo cells (uncrosslinked); lane 2, lysate of DSS crosslinked LoVo cells; lane 3, proteins pulled down by aptamer BG2 from lysate of DSS crosslinked LoVo cells.

Figure 4. In vivo and ex vivo tumor imaging stained by BG2. LoVo cell xenografted BALB/c nu/nu mice were intravenously injected with a) control sequence L45-AF647, and b) BG2-AF647; c) PC-3 cell xenografted BALB/c nu/nu mouse was intravenously injected with BG2-AF647. Up: in vivo imaging, down: ex vivo tumor imaging.
3. Discussion

As shown above, we have proved that the molecular target of aptamer BG2 is the heterodimers of IAP and PLAP/GCAP. This is the first probe that specifically recognizes a protein dimer. Aptamer BG2 folds into a duplex stabilized G-quadruplex, and the G-quadruplex is the essential motif for target binding. G-quadruplex is a four-stranded structure of nucleic acid, which is composed of planar G-quartet formed by four guanine bases stabilized by eight Hoogsteen hydrogen bonds, compared with duplex structure, G-quadruplex possesses much more variable domains that interact with adjacent molecules, such as grooves, loops, and terminal G-quartet planes. Because the size of the G-quadruplex motif of BG2 is small, it might be specifically recognized by a conserved pocket formed by IAP and PLAP. Because GCAP is highly homologous to PLAP, BG2 also bound IAP–GCAP heterodimer.

AP isozymes, such as PLAP, IAP, and GCAP, are found in variety of tissues, which are detected by corresponding monoclonal antibodies. The general function of APs is to catalyze the hydrolysis of phosphate esters. APs are associated with cell differentiation potential and stemness during development, and are reported as markers for pluripotent embryonic stem cells and cancers. For example, PLAP or IAP is reported to overexpress in breast cancer, hepatocellular carcinoma, cervical cancer, colon cancer, neoplasia as germ cell tumors, and squamous cell carcinoma of the lung. PLAP- or GCAP-producing tumors in both mice and humans. Because of the immunoassay with monoclonal antibodies, it is impossible to obtain a native probe for heterodimers, currently, it is impossible to obtain a native probe for heterodimers.

In summary, we report here a G-rich DNA aptamer, BG2 derived by cell-SELEX, that specifically recognizes IAP–PLAP/GCAP heterodimers. BG2 folds into a duplex stabilized antiparallel G-quadruplex structure, can recognize cultured cells highly expressing AP heterodimers, and can recognize tumor xenografts in nude mice. These results indicate that BG2 hold great promise in the study of AP heterodimers in vitro and in vivo. To our knowledge, this is the first single molecular probe against a heterodimer protein. Our results also show that cell-SELEX is a feasible way to generate aptameric probes toward protein dimers.

4. Experimental Section

Materials: All reagents unless otherwise noted were from Sigma-Aldrich (St. Louis, MO). Heavy lysine ([13C6, 15N2]-lysine) and arginine ([13C6]-arginine), 99% in isotopic purity and 98% in chemical purity, were purchased from Silantes GmbH (Andover, Germany). Oligonucleotides were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Washing buffer was prepared by adding 5 mmol MgCl2 and 4.5 g glucose into 1 L of phosphate buffer saline (PBS) (contain 8 g NaCl, 0.2 g KCl, 3.58 g Na2HPO4, 1.44 g KH2PO4, per 1 L ddH2O, pH = 7.4). Binding buffer was prepared by adding 1 mg mL−1 bovine serum albumin (BSA, Sigma) and 0.1 mg mL−1 herring sperm DNA (Sigma) into washing buffer.

Cell Culture: Cell lines: HeLa (human adenocarcinoma cervical cancer), MCF-7 (human breast cancer), Jurkat (clone E6-1) (human acute T lymphoblastic leukemia), HEK-293 (human embryonic kidney), and SH-SY5Y (human neuroblastoma), were purchased from the Institute of Basic Medical Science at the Chinese Academy of Medical Sciences (Beijing, China). LoVo and HCT-116 (human colorectal cancer), PC-3 (prostate cancer), SMMC-7721, and HepG2 (human liver hepatocellular carcinoma) were purchased from Shanghai Cell Bank, Chemical Academy of Sciences (Shanghai, China). Unless otherwise indicated, all cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100 IU mL−1 penicillin-streptomycin (Invitrogen). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2, with medium renewal at every 2–3 days. For stable isotope labeling with amino acids in cell culture experiments, the RPMI 1640 medium without l-lysine or l-arginine was custom-prepared following the ATCC formulation. The complete light and heavy RPMI 1640 media were prepared by the addition of light or heavy lysine and arginine, together with dialyzed FBS (PAN-Biotech Gmbh, Germany), to the above lysine, arginine-depleted medium. LoVo cells were cultured in light or heavy RPMI 1640 medium for at least six cell doublings to achieve complete isotope incorporation.
Selection of Cell Binding Aptamers: DNA library, BbIb: 5'-ACCCCTGCATGCCTACACTACGTYRRRRNNGGGNNGGGNNNGGNNGNNNNNNNNNGGNNYYRTCTGACGCTACGTCGCTCAG-3', where N = A, T, G or C; Y = C or T; R = A or G. Forward primer, BP-1:5'-FAM-ACGCCCTGATGACTACAG-3'; Reverse primer, BP-2: 5'-biotin-GTACCAGCGACTTCGATGAG-3'.

Flow Cytometric Analysis: A total of 5 x 10^5 cells were incubated with antibodies (anti-IAP, GTX60746, Gene Tex; anti-PLAP, MA1-20245, Thermo Fisher Scientific) and/or FITC- or AF647-labeled aptamers in 10 µL of binding buffer (100 x 10^-9 M) on ice for 30 min. The cells were washed once, and added with antimouse IgG-PE. After 30 min incubation, the cells were washed again, resuspended in 0.4 mL of binding buffer (100 x 10^-9 M) on ice for 30 min. Then the cells were lysed and the aptamer-target complexes were pulled down using streptavidin-coated beads. The Ap activity of the pull-down protein was measured using an Alkaline Phosphatase Assay Kit (Beyotime, China) according to the manufacturer’s instructions.

Cell Surface Anchoring of PLAP Proteins: For cell surface anchoring with GPI-linked PLAP protein, the Jurkat cells (AP nonexpressing) were washed twice, resuspended in serum-free medium at 2 x 10^6 cells mL^-1 and incubated with human PLAP protein (0.2 mg mL^-1) for 2 h at 37 °C for occasional inversion. Anchored cells were extensively washed, and the membrane insertion efficiency assessed by FACS analysis.

Transfection of APs in PC-3 Cells: The cDNA of IAP (P09923) or PLAP (P05187) constructed by PCR-based amplification of cDNA from LoVo cells was inserted into mammalian expression plasmid pCMV-myc vector with restriction endonucleases (Xho1 and EcoR1, NEB), respectively. The cDNA of GCAP (P10696) purchased from Youbio Biological Technology Co., Ltd. was inserted into mammalian expression plasmid pcDNA3.1(+) plasmid with restriction endonucleases.
subcutaneous injection of 3–6 weeks old female BALB/c nu/nu mice (obtained from the University Health Science Center. Prior to initiation of the experiments, mice were acclimatized to husbandry conditions for 1 week to eliminate stress. Four–six weeks old female BALB/c nu/nu mice were anesthetized to be motionless, a 100 µL of transfection reagent per well. Lipofectamine 3000 Transfection Reagent (Thermo Scientific) with 3 µL of plasmid and 6 µL of transfection reagent per well. After 48 h of transfection, cells were collected and stained with aptamers or antibodies as above.

Confocal Image of Cells: 2 × 10⁶ cells were cultured in covered glass-bottomed cell confocal dishes (NEST Biotechnology Co. Ltd.) for more than 1 day to get a well extension. For living cell imaging, cells were incubated with aptamers at 4 °C for 30 min. For fixed cell imaging, cells were fixed using 1% formaldehyde in PBS on ice for 10 min, and then incubated with aptamers at 4 °C for 30 min. Confocal microscopy imaging was performed with an OLYMPUS FV1000 IX81 confocal microscope (Olympus Corporation, Japan) with different objective lenses. The images were analyzed by FV10-ASW.

Western Blot Assay of Protein Dimers: 1 × 10⁷ LoVo cells were incubated with or without Biotin-disulfide-labeled aptamer BG2 on ice for 30 min in 0.5 mL of binding buffer, after which 25 µL of 100 × 10⁻³ M disuccinimidyl suberate (Thermo Fisher Scientific) were added to crosslink the protein dimers. The mixture was incubated on ice for 120 min to induce crosslinking in situ and terminated by the addition of 25 µL of 1 M Tris-HCl buffer, pH 7.0. Then, cells were lysed and the aptamer-target complexes were pulled down using streptavidin-coated beads. The membrane proteins or the aptamer-pulled-down proteins were mixed with 2.5 µL of 4× SDS sample loading buffer with 5% stacking gel; the western blot assay of the pull-down proteins was performed as above.

In Vivo and Ex Vivo Optical Imaging: All animal experiments were performed in strict compliance with the guide of the Care and Use of Laboratory Animals of Department of Laboratory Animal Science, Peking University Health Science Center. Prior to initiation of the experiments, mice were acclimatized to husbandry conditions for 1 week to eliminate the stress. 4–6 weeks old female BALB/c nu/nu mice (obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd.) received a subcutaneous injection of 3 × 10⁶ LoVo or PC-3 cancer cells into right armpit. Tumors were then allowed to grow to 0.6–1.2 cm in diameter for about 4 weeks. Before imaging, BALB/c nu/nu mice were anesthetized with the combined use of tranquilizer and anesthetic. Once the mice were anesthetized to be motionless, a 100 µL of BG2-AF647 (3 µM) or random sequence L45-AF647 (3 µM) was injected intravenously through the tail vein. After 30 min, fluorescence images of chest side of live mice were taken by a Maestro in vivo fluorescence imaging system (Cambridge Research & Instrumentation, Inc.). A 586–601 nm bandpass excitation filter and the emission filter, respectively. For ex vivo imaging, excised tumors were placed in the optical imaging system and imaged as above. The fluorescence images were presented after processing by Maestro software (version 2.10.0).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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[1] a) N. J. Marianayagam, M. Sunde, J. M. Matthews, Trends Biochem. Sci. 2004, 29, 618; b) C. H. Heldin, Cell 1995, 80, 213.
[2] a) H. Guo, S. An, R. Ward, Y. Yang, Y. Liu, X. X. Guo, Q. Hao, T. R. Xu, Biosci. Rep. 2017, 37, BS20160547; b) S. Zilkenat, I. Grin, S. Wagner, Biol. Chem. 2017, 398, 155.
[3] H. Imanishi, T. Hada, K. Muratani, K. Hirano, K. Higashino, Cancer Res. 1990, 50, 3408.
[4] a) H. Kodama, K. Asai, T. Adachi, Y. Mori, K. Hayashi, K. Hirano, T. Stigbrand, Biochim. Biophys. Acta, Gene Struct. Expression 1994, 1218, 163; b) C. M. Behrens, C. A. Enns, H. H. Sussman, Biochem. J. 1983, 211, 553.
[5] A. Wada, A. P. Wang, H. Isomoto, Y. Satomi, T. Takao, A. Takahashi, S. Awata, T. Nomura, Y. Fujii, S. Kohno, K. Okamoto, J. Moss, L. L. Millan, T. Hirayama, Int. J. Med. Microbiol. 2005, 294, 427.
[6] a) W. Zhang, Y. Jiang, Q. Wang, X. Ma, Z. Xiao, W. Zuo, X. Fang, Y. G. Chen, Proc. Natl. Acad. Sci. USA 2009, 106, 15679; b) A. D. Goddard, A. Watts, Biophys. Rev. 2012, 4, 291.
[7] a) M. Gullberg, A.-C. Andersson, Nat. Methods 2010, 7, 10; b) H. Liang, S. Chen, P. Li, L. Wang, J. Li, J. Li, H. H. Yang, W. Tan, J. Am. Chem. Soc. 2018, 140, 4156.
[8] S. Buus, J. Rockberg, B. Forstrom, P. Nilsson, M. Uhlen, C. Schafer-Nielsen, Mol. Cell. Proteomics 2012, 11, 1790.
[9] a) A. D. Ellington, J. W. Szostak, Nature 1990, 346, 818; b) C. Tuerk, L. Gold, Science 1990, 249, 505; c) J. Liu, Z. Cao, Y. Lu, Chem. Rev. 2009, 109, 1948; d) D. Shangguan, Y. Li, Z. Tang, Z. C. Cao, H. W. Chen, P. Mallikarachchi, K. Sefah, C. J. Yang, W. Tan, Proc. Natl. Acad. Sci. USA 2006, 103, 11838; e) J. H. Zhou, J. Rossi, Nat. Rev. Drug Discovery 2017, 16, 181; f) L. Civit, S. M. Taghdisi, A. Jonczyk, S. K. Hassel, C. Grober, M. Blank, H. J. Stundn, M. Beyer, J. Schultz, E. Latz, G. Mayer, Biochimie 2018, 145, 53.
[10] F. Opazo, M. Levy, M. Byrom, C. Schafer, C. Geisler, T. W. Groemer, A. D. Ellington, S. O. Rizzoli, Nat. Methods 2012, 9, 938.
[11] B. Deng, Y. W. Lin, C. Wang, F. Li, Z. X. Wang, H. Q. Zhang, X. F. Li, X. C. Le, Anal. Chim. Acta 2014, 837, 1.
[12] a) L. Q. Zhang, S. Wan, Y. Jiang, Y. Y. Wang, T. Fu, Q. L. Liu, Z. J. Cao, L. P. Qiu, W. H. Tan, J. Am. Chem. Soc. 2017, 139, 2532; b) L. Wang, T. Bing, Y. Liu, N. Zhang, L. Shen, X. Liu, J. Wang, D. Shangguan, J. Am. Chem. Soc. 2018, 140, 18066.
[13] a) N. Zhang, T. Bing, L. Y. Shen, R. S. Song, L. L. Wang, X. J. Liu, M. R. Liu, J. Li, W. H. Tan, D. H. Shangguan, Angew. Chem., Int. Ed. 2016, 55, 3914; b) Y. Wang, Y. Luo, T. Bing, Z. Chen, M. Lu, N. Zhang, D. Shangguan, X. Gao, PLoS ONE 2014, 9, e100243; c) W.-M. Li, T. Bing, J.-Y. Wei, Z.-Z. Chen, D.-H. Shangguan, J. Fang, Biomater. Sci. 2014, 35, 6998.
[14] T. Bing, D. Shangguan, Y. Wang, Mol. Cell. Proteomics 2015, 14, 2692.
[15] M. Wilhelm, J. Schlegl, H. Hahne, A. M. Cholami, M. Lieberenz, M. M. Savitski, E. Ziegler, L. Buttmann, S. Gesslatur, H. Marx, T. Mathieson, S. Lemeer, K. Schnatbaum, U. Reimer, H. Wenschuh, M. Mollenhauer, J. Slotta-Huspenina, J. H. Boese, M. Bantscheff, A. Gerstmaier, F. Faerber, B. Kuster, Nature 2014, 509, 582.
[16] a) H. Kohno, K. Sudo, T. Kanno, Clin. Chim. Acta 1983, 135, 41; b) M. Mäder, N. Kolbus, D. Meierhorst, A. Köhn, W. Beuche, K. Felgenhauer, Clin. Exp. Immunol. 1994, 95, 98; c) P. M. Crofton, Clin. Chim. Acta 1981, 112, 33.
[17] U. Sharma, D. Pal, R. Prasad, Indian J. Clin. Biochem. 2014, 29, 269.
[18] M. H. Le Du, T. Stigbrand, M. J. Taussig, A. Menez, E. A. Stura, J. Biol. Chem. 2001, 276, 9158.
[19] E. Ruggiero, S. N. Richter, Nucleic Acids Res. 2018, 46, 3270.
[20] a) M. D. O’Connor, M. D. Kardel, I. Iosfina, D. Youssef, M. Lu, M. M. Li, S. Vercauteren, A. Nagy, C. J. Eaves, Stem Cells 2008, 26, 1109; b) K. Stefkova, J. Prochazkova, J. Pachernik, Stem Cells Int. 2015, 2015, 1.
[21] a) L. C. Tsai, M. W. Hung, Y. H. Chen, W. C. Su, G. G. Chang, T. C. Chang, Eur. J. Biochem. 2000, 267, 1330; b) J. Shin, A. Carr, G. A. Corner, L. Togel, M. Davalos-Salas, H. Tran, A. C. Chueh, S. Al-Obaidi, F. Chionh, N. Ahmed, D. D. Buchanan, J. P. Young, M. S. Malo, R. A. Hodin, D. Arango, O. M. Sieber, L. H. Augenlicht, A. S. Dhillon, T. K. Weber, J. M. Mariadason, J. Biol. Chem. 2014, 289, 25306; c) J. L. Millan, W. H. Fishman, Crit. Rev. Clin. Lab. Sci. 1995, 32, 1.
[22] K. Tsukazaki, E. G. Hayman, E. Ruoslahti, Cancer Res. 1985, 45, 1834.
[23] H. Imanishi, T. Hada, K. Muratani, K. Hirano, K. Higashino, Clin. Chim. Acta 1990, 186, 309.
[24] J. L. Millán, Mammalian alkaline phosphatases: from biology to applications in medicine and biotechnology, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany 2016, p. 187.