Microarray-Formatted Clinical Biomarker Assay Development Using Peptide Aptamers to Anterior Gradient-2[†]

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ABSTRACT: Anterior gradient-2 protein was identified using proteomic technologies as a p53 inhibitor which is overexpressed in human cancers, and this protein presents a novel pro-oncogenic target with which to develop diagnostic assays for biomarker detection in clinical tissue. Combinatorial phage−peptide libraries were used to select 12 amino acid polypeptide aptamers toward anterior gradient-2 to determine whether methods can be developed to affinity purify the protein from clinical biopsies. Selecting phage aptamers through four rounds of screening on recombinant human anterior gradient-2 protein identified two classes of peptide ligand that bind to distinct epitopes on anterior gradient-2 protein in an immunoblot. Synthetic biotinylated peptide aptamers bound in an ELISA format to anterior gradient-2, and substitution mutagenesis further minimized one polypeptide aptamer to a hexapeptide core. Aptamers containing this latter consensus sequence could be used to affinity purify to homogeneity human anterior gradient-2 protein from a single clinical biopsy. The spotting of a panel of peptide aptamers onto a protein microarray matrix could be used to quantify anterior gradient-2 protein from crude clinical biopsy lysates, providing a format for quantitative screening. These data highlight the utility of peptide combinatorial libraries to acquire rapidly a high-affinity ligand that can selectively bind a target protein from a clinical biopsy and provide a technological approach for clinical biomarker assay development in an aptamer microarray format.

Functional proteomics is expanding as a mass spectrometric-facilitated technology that can identify novel protein mediators of human diseases including cancer (*1*−*4*). The high-throughout proteomics approach can identify a large number of differentially expressed proteins from a sample pair, but it is still limited by the relatively slow generation of antibodies required for validation of the target protein in clinical material. Although antibodies have classically been used for target validation, methodologies that enhance the speed of multiple biomarker detection from a clinical screen would promote a more comprehensive validation of potential biomarker hits. Phage-scFv libraries have the capacity to offer significant enhancement in the speed of antibody acquisition, but require substantial downstream manipulation including mutagenesis for improvement.

Protein−protein interactions have been thought classically to require a relatively large interface to stabilize protein complex formation; however, it has been recently highlighted that many protein interactions occur over relatively short linear interaction motifs (*5*). This property might be exploited to develop small polypeptide ligands that trap target protein complexes and thus would complement the use of standard antibody technologies to selectively target a biomarker. The use of combinatorial phage−peptide libraries has the potential in theory to rapidly acquire a high-affinity ligand toward a target protein that can be used to validate protein expression in clinical material. A variety of peptide aptamer approaches can be used to selectively acquire a high-affinity peptide ligand that can bind stably to a target protein. The two most common approaches have been the in vivo yeast-based two-hybrid analysis and the in vitro phage−peptide technology. The in vivo approach has the attraction of only requiring the cloning of the target gene into a suitable vector for two-hybrid screening, while it is limited in that more sophisticated effects of ligand binding on protein conformation cannot be developed. The in vitro approach has the advantage of being able to manipulate the bait protein conformation with allosteric cofactors (*6*, *7*), while the in vitro approach is limited by the time required for purifying recombinant or native protein. The combination of both technologies would give a comprehensive panel of peptide aptamers to a target protein that can bind targets in a cellular milieu and in vitro solid-phase format.

Advances in cancer biology would be facilitated by a more widespread exploitation of peptide aptamer technologies to quantify and manipulate the cancer proteome. Although much

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progress has been achieved in understanding fundamental principles underlying the molecular basis of cancer (*8*), the knowledge is limited to only a few cancer types that are accessible to biopsy and those which are widespread such as colorectal or breast cancers. Further, this knowledge has not been widely exploited to diagnostic assays for early detection of cancers; one of the few successes is the immunochemical detection of the estrogen receptor using monoclonal antibodies and the use of monoclonal antibody therapies to the ErbB receptor in breast cancers (*26*). Since organ-specific and local microenvironmental factors likely mediate cancer initiation and progression as a tissue-specific disease, there are likely tissue-specific factors that would serve as novel diagnostic or therapeutic targets for the disease. The development of methodologies to allow the application of functional proteomics to a range of human diseases will help to develop better diagnostic assays and a more thorough understanding of the mechanisms underlying disease development. As such, we have been interested in developing clinical-proteomic models in human cancer and have first focused on models where selection pressures are placed on the tumor suppressor p53 pathway.

The p53 gene is mutated in most human cancers, and the gene product acts at a key nodal point in the cellular stress response identifying a key pathway that is likely to be a target of tissue-specific inhibitors in the carcinogenic sequence (*9*). The selective pressures that drive mutations in the tumor suppressor gene p53 in human cancers are not clear, but are thought to include hypoxia or anoxia in colon cancers and acid bile reflux associated DNA damage in the case of esophageal adenocarcinoma (*10*−*12*). The esophageal carcinogenesis sequence evolves from normal squamous epithelium to metaplastic epithelium to dysplasia and onto adenocarcinoma. This multistage cancer progression model therefore provides an opportunity to define novel stress-protein responses. One novel and uncharacterized stress-responsive protein we identified in normal squamous epithelium by a functional proteomics approach was a protein named SEP53 (*13*): this protein was identified as a novel stress protein whose synthesis increased using ex vivo organ culture, which was subsequently shown to function as an antiapoptotic modifier (*14*), and which has a genetic signature of a gene undergoing rapid adaptive evolution (*29*). Having characterized the novel stress responses in normal esophageal squamous epithelium, we went on to develop a proteomics approach to identify proteins upregulated in esophageal metaplasia (*3*), a preneoplastic condition (called Barrett's epithelium) where selection pressures for p53 mutation exist and a condition which increases the risk of adenocarcinoma of the esophagus (*15*). In this proliferative disease, p53 is mutated in 20−70% of the metaplasias, indicating that 30−80% of the metaplasias maintain the wt-p53 gene (*16*, *17*). The pressures for either mutating p53 or maintaining the wt-p53 gene are not clear, but this is a potentially fertile area to identify novel p53 modifiers. This proteomics approach was successful in identifying a protein named anterior gradient-2 (AG-2), the most abundant and novel protein upregulated in the proliferative Barrett's epithelium (*3*).

Although anterior gradient-2 is currently not embedded in a known pathway, it can inhibit the p53-dependent response to DNA damage (*3*) and can mediate metastasis in animal models (*18*). As such, we have focused attention on anterior gradient-2 function to dissect in more detail the anterior gradient-2 dependent proteome, to identify the function and regulation of anterior gradient-2 to expand its pathway, to evaluate its potential as an anticancer drug target, and to develop diagnostic assays for anterior gradient-2 protein detection. This latter aim was achieved in this paper, where we identify using combinatorial peptide libraries high-affinity peptide aptamers that can be used to affinity purify anterior gradient-2 protein from a single clinical biopsy. This enabling technology will facilitate the development of biomarker assays for the detection of anterior gradient-2 protein in clinical material and highlights the utility of combinatorial peptide libraries to rapidly acquire a ligand for purification of a protein for cancer biomarker development in an aptamer microarray format.

EXPERIMENTAL PROCEDURES

Peptide−*Phage Display Biopanning.* Chemicals were obtained from Sigma unless otherwise indicated. Recombinant human His-tagged anterior gradient-2 (15 μg), purified as described previously (*3*), in 150 μL of coating buffer (0.1 M $NaHCO_3$, pH 8.6) was added to a well of a 96-well plate and incubated overnight at 4 °C. The following day the coating buffer was removed and the well was blocked with blocking buffer (5 mg/mL BSA in $NaHCO_3$, pH 8.6) for 1 h at 4 °C followed by six washes with 200 μL of TBST (Tris (pH 8.0) buffered saline + 0.1% (v/v) Tween-20). A phage display library kit (New England Biolabs; 10 μL of or 4×10^{10} phages; *7*) was diluted in 100 μL of TBST, added to the well, and incubated at room temperature for 1 h. Nonbinding phage particles were removed, and the well was washed six times with TBST. Binding phages were then eluted with 100 μL of 0.2 M glycine−HCl, pH 2.2, containing 1 mg/mL BSA by incubating at room temperature for 10 min, the eluate was neutralized with 15 μL of 1 M Tris−HCl (pH 9.1), and a small amount of this eluate was titered and the remainder amplified. The phages were amplified by infection of ER2738 cells for 4.5 h, precipitated with PEG (20% (w/v) polyethylene glycol-8000, 2.5 M NaCl), and resuspended in 200 μL of TBS. This biopanning procedure was repeated a further three times using the amplified phage, and a small amount of eluate from each round was titered.

Characterization of Anterior Gradient-2 Binding Peptide Aptamers. From the phage titers carried out for each round of biopanning, individual plaques were isolated and amplified by infection of ER2738 cells for 4.5 h. Amplified phages were then precipitated with PEG and resuspended in 100 μL of iodine buffer (10 mM Tris−HCl, pH 8.0, 1 mM EDTA, 4 M NaI), and their DNA was precipitated with 250 μL of ethanol. After thorough washing, the DNA was amplified and prepared for sequencing using the Big Dye terminator (Amersham Biosciences) kit.

Western Blot Detection of Anterior Gradient-2 Using Phage Peptides. SDS−PAGE of recombinant His-tagged anterior gradient-2 was performed in the usual manner and blotted onto HighBond C membrane. The membrane was blocked with 3% milk in PBST (PBS + 0.5% (v/v) Tween-20) for 1 h and rinsed with PBST. Phage particles were diluted in 3% BSA in PBST (1:100) and added to the membrane overnight at 4 °C, and the membrane was washed

with PBST for 3 × 10 min. Binding was detected using HRP-conjugated anti-M13 antibody (Pharmacia, no. 27-9421-01) and developed with ECL.

Western Blot Detection of Anterior Gradient-2 Using Synthetic Biotinylated Peptides. SDS−PAGE of either recombinant His-tagged anterior gradient-2 or pig stomach lysate was performed in the usual manner and blotted onto HighBond C membrane. The membrane was blocked with 3% milk in PBST for 1 h and rinsed with PBST. Biotinylated peptides were first dissolved in DMSO (5 mg/mL), then further diluted 1:1000 in 3% BSA in PBST, and added to the membrane for 1 h at room temperature. The membranes were washed with PBST for 3 × 10 min, streptavidin peroxidase conjugate (0.5 mg/mL) diluted 1:1000 in 3% BSA in PBST was added, and the membranes were washed again with PBST for 3 × 10 min before detection using ECL.

ELISA Quantification of the Binding of Anterior Gradient-2 to Synthetic Peptides. The wells of an ELISA plate were coated with 50 μL of streptavidin (2 mg/mL) overnight at 37 °C. The following day the wells were washed four times with 200 μL of PBST, 50 μL of peptide (0.1 mg/mL) was added for 1 h at room temperature, and the wells were washed six times with 200 μL of PBST and blocked with 200 μL of 3% BSA in PBST for 1 h. A dilution series of either recombinant His-tagged anterior gradient-2 or porcine stomach lysate was prepared and added to the wells either for 1 h at room temperature or overnight at 4 °C. The wells were then washed six times with PBST and probed for the presence of anterior gradient-2 using polyclonal anti-anterior gradient-2 antibody diluted 1:2000 in blocking buffer. The reaction was detected using swine anti-rabbit HRP monoclonal antibody diluted 1:2000, developed by ECL, and read using Fluoroskan Ascent FL. The polyclonal antibody to anterior gradient-2 was raised in rabbit immunized with recombinant protein by Moravian Biotechnology Ltd., Brno, Czech Republic.

Lysis of Tissue. Normal porcine gastric epithelium was obtained from Dr. Tim King, University of Edinburgh. Esophageal normal squamous or long-segment Barrett's epithelial biopsies and corresponding cell lysates were obtained and stored from a previous study (*3*). These samples were collected into PBS prechilled at 0 °C and frozen in liquid nitrogen to examine the steady-state pattern of protein synthesis using Coomassie-stained SDS−polyacrylamide gels. Epithelial samples from porcine or human origin were lysed by homogenizing in lysis buffer (0.5% Triton X-100, 50 mM HEPES (pH 7.6), 1 mM DTT, 0.4 M KCl, 5% glycerol, 5 mM EDTA, 50 mM NaF, 10 mM β-phosphoglycerate, 2 μg/mL Pefabloc (Boehringer-Mannheim), and a Complete (1×) protease inhibitor mixture from Calbiochem) and incubated at 0 °C for 15 min (*19*). Soluble supernatant was obtained after centrifugation at 14 000 rpm in a minicentrifuge at 2 °C for 10 min, and protein concentrations were determined by the method of Bradford.

Affinity Purification of Anterior Gradient-2 Protein Using Synthetic Peptides. Synthetic peptides containing an N-terminal biotin and SGSG linker were obtained from Alta Biosciences (Birmingham, U.K.) and Mimotopes (Clayton Victoria, Australia). To streptavidin immobilized on 4% beaded agarose (100 μL) which had been washed once with PBST (200 μL) and resuspended in PBST (200 μL) was added 4.5 μL of peptide (5 mg/mL), and the mixture was incubated at 4 °C overnight on a revolving wheel. The following day the gel was centrifuged for 2 min at 5000 rcf, the supernatant was removed, and the gel was then washed six times with PBST (200 μL) by vortexing and then centrifuging as before. Lysates were added to the matrix, and the mixture was incubated at 4 °C overnight on a revolving wheel. The following day the lysates were removed and the gel was washed as before. To elute bound protein, 50 μL of 0.1 M glycine−HCl, pH 2.5, was added and the mixture incubated at room temperature on a rotating wheel for 10 min. The eluate was removed, neutralized with 5 μL of 1.5 M Tris−HCl, pH 8.8, and analyzed by SDS−PAGE.

Aptamer Microarrays. (1) Reagents. Streptavidin-coated slides used to manufacture arrays were superflat high-quality coating SuperStreptavidin slides from TeleChem ArrayIt. Peptide aptamers supplied for the array manufacture were at a starting concentration of ∼5 mg/mL in DMSO. Probe protein AG-2 was supplied at 0.6 mg/mL in its elution buffer. Anti-rabbit AlexaFluor647 conjugate was purchased from Invitrogen. For the analysis of crude tissue lysates containing AG-2, porcine stomach lysate was at ∼9 mg/mL total protein and Barrett's epithelial biopsy lysate was at ∼2 mg/mL.

(2) Protocols. (a) Aptamer Spotting Stage. Arraying protocols were created on a Perkin-Elmer Piezorray using built-in software. Slides were arrayed with 500 μm spacing between spots with a spot volume of 1 nL. Spotting buffer was PBS with 10% DMSO with an arraying concentration of aptamers of ∼0.5 mg/mL. Up to eight array pads were normally created on a slide. The temperature of the clean room during arraying was 20 ± 0.2 °C at 43−47% relative humidity (RH), and the source plate was chilled to 10 °C. After the arrays were spotted, they were allowed to incubate overnight on the deck of the arrayer in the clean room.

(b) Blocking Stage. Blocking of the array was performed with SuperStreptavidin blocker solution from TeleChem for 1 h at room temperature. After blocking the arrays were air-dried.

(c) Probe Incubation Stage. For probing, purified AG-2 protein or crude lysates were prepared in probe buffer (PBS containing 3% BSA) at the desired concentrations and dilutions. Probing frames were placed on top of the blocked dry slide containing the array, ensuring that the wells fit around the array pads containing a full array each, and assembled appropriately for the type of frame. In the course of this work well frames FlexWell from Grace Bio Labs and SIMPlex from GenTel Biosciences were used. A 70 μL aliquot of probe solution was added to each well. After application of the probe to the wells at the desired concentrations and sealing of the frames with a strip of Mylar plate sealer, the reaction was allowed to incubate for 1 h at room temperature. After incubation with the probe, the sealer strip was removed and the contents of the wells were carefully aspirated with a pipet using a fresh tip for every sample. The postprobe wash involved three wash cycles, each cycle consisting of filling the wells with PBST, followed by 10 min of soaking with agitation on a horizontal shaker, and subsequent removal of the contents of the wells by turning the frame upside down to shake the solution off.

(d) Probe Detection Stage. Primary anti-AG-2 antibody solution was applied at 1:1000 in probe buffer to all reaction wells (70 μL), the reaction wells were sealed, and incubation was continued for 1 h at room temperature. After the

1	HWDPFSLSAYFP
2	TPGNPHTPNVGP
3	YPWHHSWTHTTL
4	YPWHHSWTHTTL
5	HTLTTIFGQAVP
6	YPWHHSWTHTTL
7	PHWPLYTPPASP
8	YPWHHSWTHTTL
9	YPWHHSWTHTTL
10	HLPTTIYYGPPG
11	HTLTTIFGQAVP
12	HTLTTIFGQAVP
13	YPWHHSWTHTTL
14	YPWHHSWTHTTL
15	YPWHHSWTHTTL
16	HTLTTIFGQAVP
17	YPWHHSWTHTTL
18	YPWHHSWTHTTL

FIGURE 1: Isolation of peptide aptamers to recombinant human anterior gradient-2. Recombinant human anterior gradient-2 purified from *E. coli* (15 μg) was adsorbed to a solid phase and presented to the phage−peptide library. After each round of selection, up to 20 peptide clones were sequenced and scored for similarity (as indicated by color coding). Peptide sequences from round 4 are depicted, and representative peptides from round 2−4 were used for quantitative array binding, as summarized in Figure 7.

incubation the liquid was carefully aspirated and washed in PBST by the same method as described for the postprobe wash. After the last wash the secondary fluorescently labeled antibody was added at 1:1000 in probe buffer followed by 1 h of incubation in the dark and a three-cycle wash in PBST. After the final wash the arrays were air-dried.

(e) Imaging and Quantitation of Arrays. The arrays were imaged on Perkin-Elmer ScanArray Express using a 633 nm laser excitation wavelength and an AlexaFluor647 filter set. The analysis was performed using the quantitate function within the ScanArray Express software, and the numbers presented are the spot mean minus the background expressed as relative fluorescence units (RFU).

RESULTS

Peptide Aptamer Selection Process. Recombinant human anterior gradient-2 protein was purified from *Escherichia coli* expression vectors and used as a target in a screen for high-affinity peptide binding ligands using a peptide−phage combinatorial library. Anterior gradient-2 protein (15 μg) was adsorbed onto a solid phase, a combinatorial peptide−phage library was added, and unbound peptides were removed by washing. Peptides bound selectively to anterior gradient-2 protein were released using low-pH (2.5) glycine, the samples were neutralized to pH 7.5, and the peptides were amplified by virtue of their linkage to bacteriophages that can be infected and grown in *E. coli*. These selection and amplification processes were repeated four times until related polypeptide sequences were isolated, indicating that the amplification and selection process from the random library reached termination.

By the fourth round of selection, we observed the stable enrichment of two distinct repeat peptides (Figure 1): one of the sequences is YPWHHSWTHTTL (highlighted in red), and the other class gives rise to a consensus site core of HxxTTI(F/Y) (highlighted in green and blue). Individual

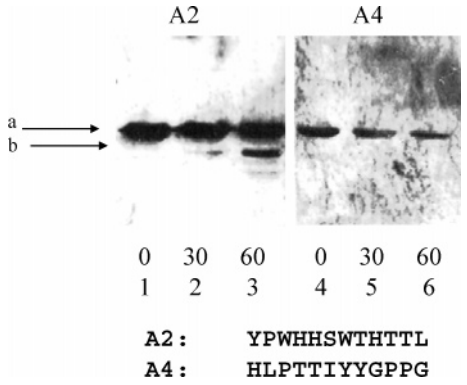


FIGURE 2: Peptide−phage immunoblotting of anterior gradient-2 protein. Epitope mapping of the bioactive peptide aptamers. Recombinant human anterior gradient-2 protein was trypsinized partially (0, 30, or 60 min) and immunoblotted with the peptide aptamers (A2 and A4). Peptide A2 detected a proteolytic fragment (lanes 1−3), while peptide A4 had an epitope within a trypsin site, since the binding is lost with increasing trypsinization (lanes 4−6).

clones ϕA2 and ϕA4, representing these two classes of peptides, were purified and used for immunoblotting. To test whether the peptides bind to different regions on anterior gradient-2 protein, the protein was partially cleaved by trypsin to fragment the protein. This partially fragmented protein was then used in an immunoblot to determine whether the two distinct peptides bound to the same region. The peptide ϕA4 (HxxTTI(F/Y) core) bound to uncleaved anterior gradient-2 (Figure 2, lane 4), while the epitope on anterior gradient-2 for this peptide aptamer was sensitive and attenuated by the trypsinization (Figure 2, lanes 5 and 6). However, the unrelated peptide ϕA2 (YPWHHSWTHTTTL) bound to a partially cleaved lower molecular mass fragment of anterior gradient-2 (Figure 2, lanes 2 and 3 vs lane 1). These data indicate that the ϕA2 and ϕA4 peptide aptamers bind to different regions on the target protein and indicate that it is possible to acquire epitope specificity using the peptide aptamer approach.

Two peptide aptamers representing the two classes of aptamers (A2 and A4) were synthesized with an N-terminal biotin and SGSG linker and used to determine whether anterior gradient-2 protein could bind to the aptamers when separated from the bacteriophage scaffold. This would simplify its use as a ligand for a variety of assays. When biotinylated peptides YPWHHSWTHTTTL (A2) and HLPTTIYYGPPG (A4) were captured onto the solid phase, a dose-dependent binding of anterior gradient-2 could be detected with linear dose-dependent saturation at approximately 160 ng (Figure 3A). Increasing levels of anterior gradient-2 protein binding may reflect a second binding reaction that might involve an unfolding stage in the anterior gradient-2 protein−peptide interaction. The HTLTTIFGQVAP peptide (A3) surprisingly bound only marginally above the control peptide (Figure 3A), suggesting that the mutations without the HxxTTI(F/Y) core destabilized the binding to anterior gradient-2 protein. A titration of the A2 and A4 peptide aptamers with a fixed amount of anterior gradient-2 protein demonstrated further those biotinylated peptides YPWHHSWTHTTTL (A2) and HLPTTIYYGPPG (A4) bound more avidly to anterior gradient-2 protein than other peptides (Figure 3B). At lower peptide aptamer concentrations, the A4 aptamer (HLPTTIYYGPPG) bound more avidly than the A2 aptamer (Figure 3B). This contrasts with the better

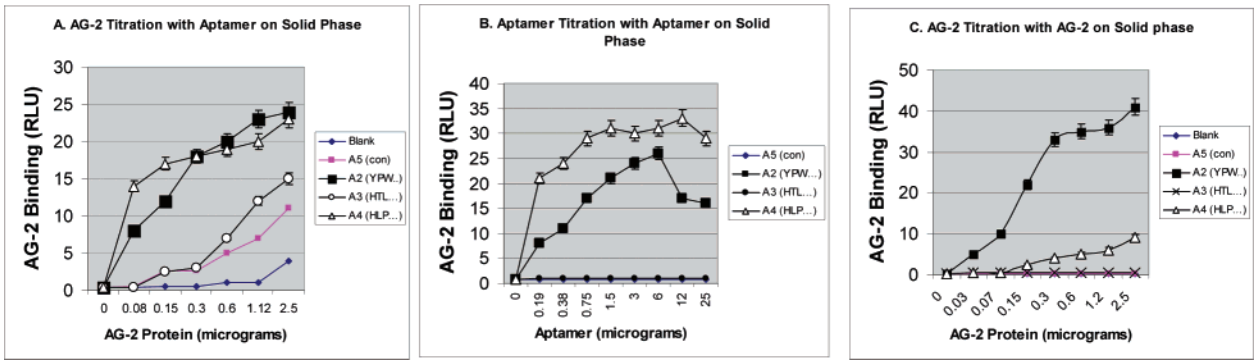


FIGURE 3: Peptide aptamers bind recombinant anterior gradient-2 in an ELISA. (A) Anterior gradient-2 protein titration using peptide capture. Increasing amounts of recombinant human anterior gradient-2 protein were titrated into ELISA wells precoated with the indicated biotinylated peptide (5 μg/well), and anterior gradient-2 bound was quantified using a primary polyclonal antibody to anterior gradient-2 and secondary antibody coupled peroxidase. Binding is indicated as relative light units. (B) Anterior gradient-2 protein capture using peptide titration. Increasing amounts of biotinylated peptide (as indicated, A2, A3, A4, or A5 control) were titrated into ELISA wells precoated with streptavidin, and the binding of fixed amounts of recombinant human anterior gradient-2 protein (160 ng) was quantified using a primary polyclonal antibody to anterior gradient-2 and secondary antibody coupled peroxidase. Binding is indicated as relative light units. (C) Anterior gradient-2 protein binding activity when adsorbed directly onto the solid phase. Increasing amounts of recombinant human anterior gradient-2 protein were titrated into ELISA wells, followed by incubation with the indicated biotinylated peptide (2 μg/well) and streptavidin peroxidase. Anterior gradient-2 bound was quantified by measuring peroxidase activity. Binding is indicated as relative light units.

binding affinity of the A2 aptamer in the denaturing immunoblot assay (Figure 2).

We finally tested the ability of the aptamers to bind to anterior gradient-2 when the protein was adsorbed onto the ELISA well, which is the original method used for phage−peptide selection. When the target protein was first captured onto the solid phase, aptamer A4 bound much weaker than the A2 aptamer (Figure 3C), which contrasts with the better binding of the A4 aptamer when it is in the solid phase (Figure 3B). These data suggest that the A2 aptamer binds to anterior gradient-2 protein better when the protein is partially denatured or unfolded (a likely effect of adsorbing the protein onto the solid phase). By contrast, the A4 aptamer binds better to anterior gradient-2 protein (Figure 3B) when the protein is native. This further suggests that the A2 and A4 aptamers bind to distinct epitopes on anterior gradient-2 protein and establishes that at least two of the peptide aptamers can bind stably to anterior gradient-2 protein when the peptides are separated from the phage coat protein scaffold.

Critical contacts in peptide A4 required for the interaction with anterior gradient-2 were defined using the ELISA. Alanine scan mutagenesis highlighted key amino acids required for stable binding (Figure 4A) and formation of the core TxIYY. For example, the binding of the wt-aptamer peptide 45 is destabilized completely by mutation of T−A (peptide 49) and I−A, Y−A, and Y−A (peptides 51−53, respectively). A further truncation series (Figure 4B) indicated that six amino acids were required to form the binding interface and include (P/A)TxIYY. For example, the binding of PTTIYY (peptide 4) was destabilized by removal of the P (peptide 5) or the Y (peptide 6). Using the small untagged PTTIYY peptide in a competition assay that measures anterior gradient-2 binding to biotinylated A4 peptide on the solid phase (Figure 4C), the six amino acid peptide was able to compete as effectively as the full-length 12 amino acid peptide aptamer.

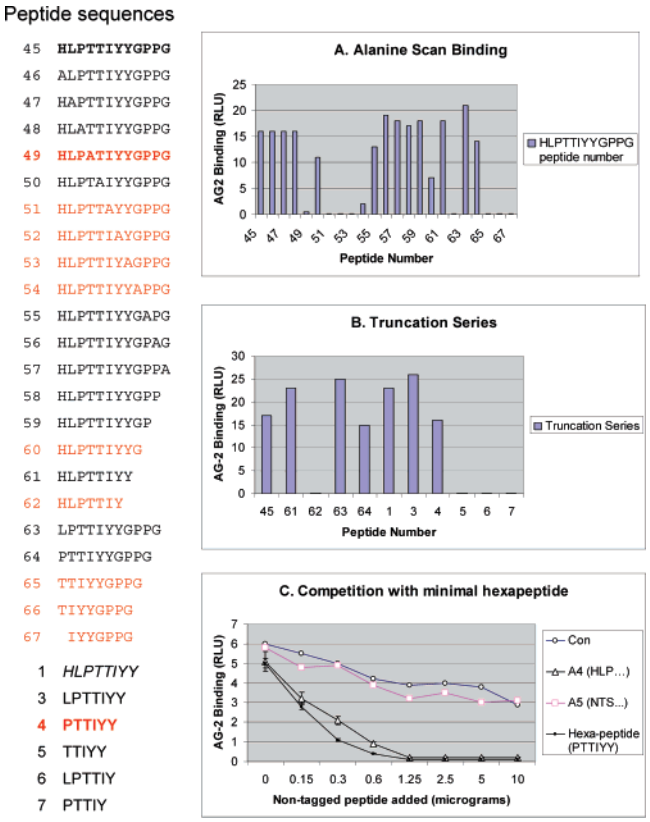


FIGURE 4: Definition of a minimal bioactive peptide sequence for peptide A4. (A, B) Peptide aptamer mutagenesis. A series of biotinylated peptides (A, alanine scan mutagenesis; B, truncation mutagenesis; as indicated in peptide sequences 1−7 and 45−67 in the left panel) were adsorbed onto streptavidin-coated wells, and AG2 binding was quantified as indicated in Figure 3. (C) Competition with the minimal hexapeptide core. Competition binding of AG2 to biotinylated peptide A4 was performed with increasing amounts of untagged peptide, as indicated: A4, A5 (control), and the minimal hexapeptide (PTTIYY). AG2 binding was quantified as indicated in Figure 3.

Affinity Purification of Anterior Gradient-2 to Near Homogeneity from a Single Clinical Biopsy on an Aptamer Column. Using this aptamer peptide-binding assay, where aptamer is linked to the solid phase and the target protein is native, lysates from tissue overexpressing anterior gradient-2 protein were screened to determine whether the peptides

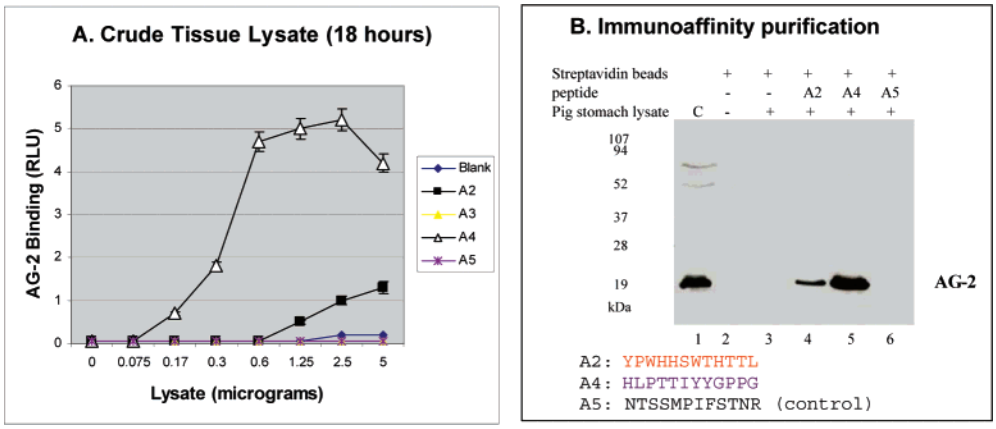


FIGURE 5: The peptide aptamer binds anterior gradient-2 protein expressed in porcine gastric epithelium. (A) Increasing amounts of pig stomach tissue lysate containing anterior gradient-2 were titrated into ELISA wells precoated with the indicated biotinylated peptide (5 μg/well) for 18 h, and anterior gradient-2 bound was quantified using a primary polyclonal antibody to anterior gradient-2 and secondary antibody coupled peroxidase. Binding is indicated as relative light units. (B) Affinity purification of anterior gradient-2 from porcine epithelium. Lysates (lane 1) were applied to an A2, A4, or A5 peptide column (lanes 4−6), bound protein was eluted with pH 2.5 glycine and loaded onto an SDS gel, and the samples were immunoblotted to quantify anterior gradient-2 protein levels.

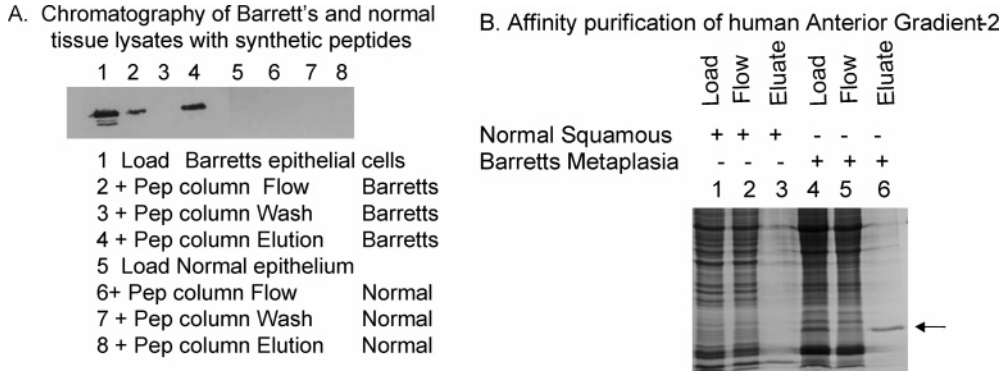


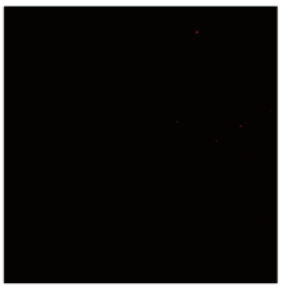
FIGURE 6: Single-step affinity purification of anterior gradient-2 protein from a single Barrett's epithelial biopsy. (A) Immunochemical detection of anterior gradient-2 during affinity purification. An A4 peptide column was incubated with Barrett's lysate (lanes 1−4) or normal squamous epithelial lysates (lanes 5−8), as indicated. The flow through was collected, and after washing with PBST buffer, bound protein was eluted with pH 2.5 glycine and loaded onto an SDS gel. The samples were immunoblotted with antibodies to anterior gradient-2 to show selective retention on the peptide column. (B) Coomassie Blue detection of anterior gradient-2 during affinity purification. An A4 peptide column was incubated with Barrett's lysate (lane 4) or normal squamous epithelial lysates (lane 1), as indicated. The flow through (lanes 2 and 5) was collected, and after washing with PBST buffer, bound protein (lanes 3 and 6) was eluted with pH 2.5 glycine and loaded onto an SDS gel. The gels were stained with Coomassie Blue to identify total protein in each lane.

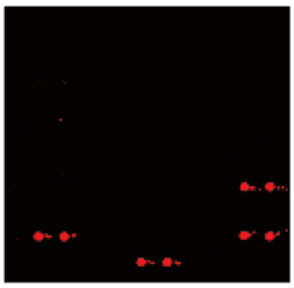
could be used to affinity purify anterior gradient-2 protein when native protein was targeted in crude tissue lysates and to define the extent of purification. Porcine gastric epithelium expresses high levels of anterior gradient-2 mRNA (unpublished data), and we evaluated whether lysates from gastric epithelium cross-react with human anti-anterior gradient-2 serum. If so, this tissue would provide an abundant source of material for optimizing the conditions required to affinity purify anterior gradient-2 protein from clinical biopsies. When lysates were titrated into ELISA wells coated with the A2, A4, and other control peptides, and incubated to allow binding to the peptide, only peptides A2 and A4 could bind to a porcine antigen that cross-reacts with the human anterior gradient-2 antiserum (Figure 5A). Further, as seen with recombinant human anterior gradient-2 protein, the aptamer A4 bound with a higher relative affinity to native porcine anterior gradient-2 protein. To determine whether the A4 peptide aptamer could be used to purify anterior gradient-2 protein from lysate as an affinity matrix, a streptavidin column was developed that contains the biotinylated peptide for use in determining whether affinity enrichment was possible. Using an 18 h incubation to ensure maximal binding of porcine anterior gradient-2 protein to the A4 peptide column, selective enrichment of anterior gradient-2 protein from crude lysates was observed (Figure 5B, lane 5 vs lane 6). Peptide A2 could enrich anterior gradient-2 to a lesser extent (Figure 5B, lane 4), consistent with its lower binding using ELISA. After the lysates were incubated with the peptide control (Figure 5B, lane 6) no anterior gradient-2 protein was observed.

Having established methods for the enrichment of porcine anterior gradient-2 protein from gastric epithelium using the peptide column, we determined whether native human anterior gradient-2 protein could be affinity purified from Barrett's epithelial lysates obtained from a single clinical biopsy. Protein from Barrett's biopsies containing anterior gradient-2 protein loaded onto the A4 aptamer column (Figure 6A, lane 1) could be eluted with the low-pH glycine buffer after a low-salt wash (Figure 6A, lanes 2−4). By contrast, normal epithelial cells did not express anterior gradient-2 protein, nor was any protein eluted from the peptide column (Figure 6A, lanes 5−8). Coomassie Blue staining of the representative lysates also demonstrated a selective depletion of anterior gradient-2 protein from the

A. Array Layout

Buf	A1	A2	A3	A4
A5	A6	A7	A8	A9
A10	A11	A12	B1	B2
B3	B4	B5	B6	B7
B8	B9	B10	B11	B12
C1	C2	C3	C4	C5
C6	C7	C8	C9	C10
C11	C12	D1	D2	D3
D4	D5	D6	D7	D8
D9	D10	D11	D12	Buf





A1: ALPRYALRPRAG	B1: FDLNPRSLYGSL	C1: TPFPFAPLGRPP	D1: TKPPVGPYWHYR
A2: TMYTQTPLRVLP	B2: NHVHRMHATPAY	C2: WPHATTPQGRWS	D2: TNLNPHLFSLIA
A3: KLFSLQVHPLPP	B3: HSSWPRHLDPPQ	C3: SYTFTMKMTQTN	D3: QMASKLVSAPTL
A4: FPWLPRDNHTLN	B4: QDVHLTQQSRYT	C4: LTPAFPNDLRVN	D4: HLPTTIYYGPPG
A5: HWDPFSLSAYFP	B5: SHPRNAQRELSV	C5: SSTINYNRLNLH	D5: YPWHHSWTHTTL
A6: HSSWYIQHFPPL	B6: HTLTTIFGQAVP	C6: SQHSHWYTKQHP	D6: NTSSMPIFSTNR
A7: VKTPLIADTTTL	B7: VTGVSLFNTMVA	C7: TPGNPHTPNVGP	D7: HTLTTIFGQAVP
A8: STHHRHYHDTLA	B8: FRPAVHNMPSLQ	C8: YPWHHSWTHTTL	D8: HLPTTIYYGPPG
A9: MPLELTPWGSDL	B9: YPSAPPQWLTNT	C9: PHWPLYTPPASP	D9: YPWHHSWTHTTL
A10: QQSMASIYFTVT	B10: SHPWNAQRELSV	C10: HLPTTIYYGPPG	D10: NTSSMPIFSTNR
A11: SGHQLLLNKMPN	B11: SLHNTSFQGSRM	C11: NTSSMPIFSTNR	D11: HLPTTIYYGPPG
A12: SAMKTAMPSHRF	B12: NWNAGKGTMSPP	C12: DRTQGTTTSYDS	D12: GPAKFGPAKFGG

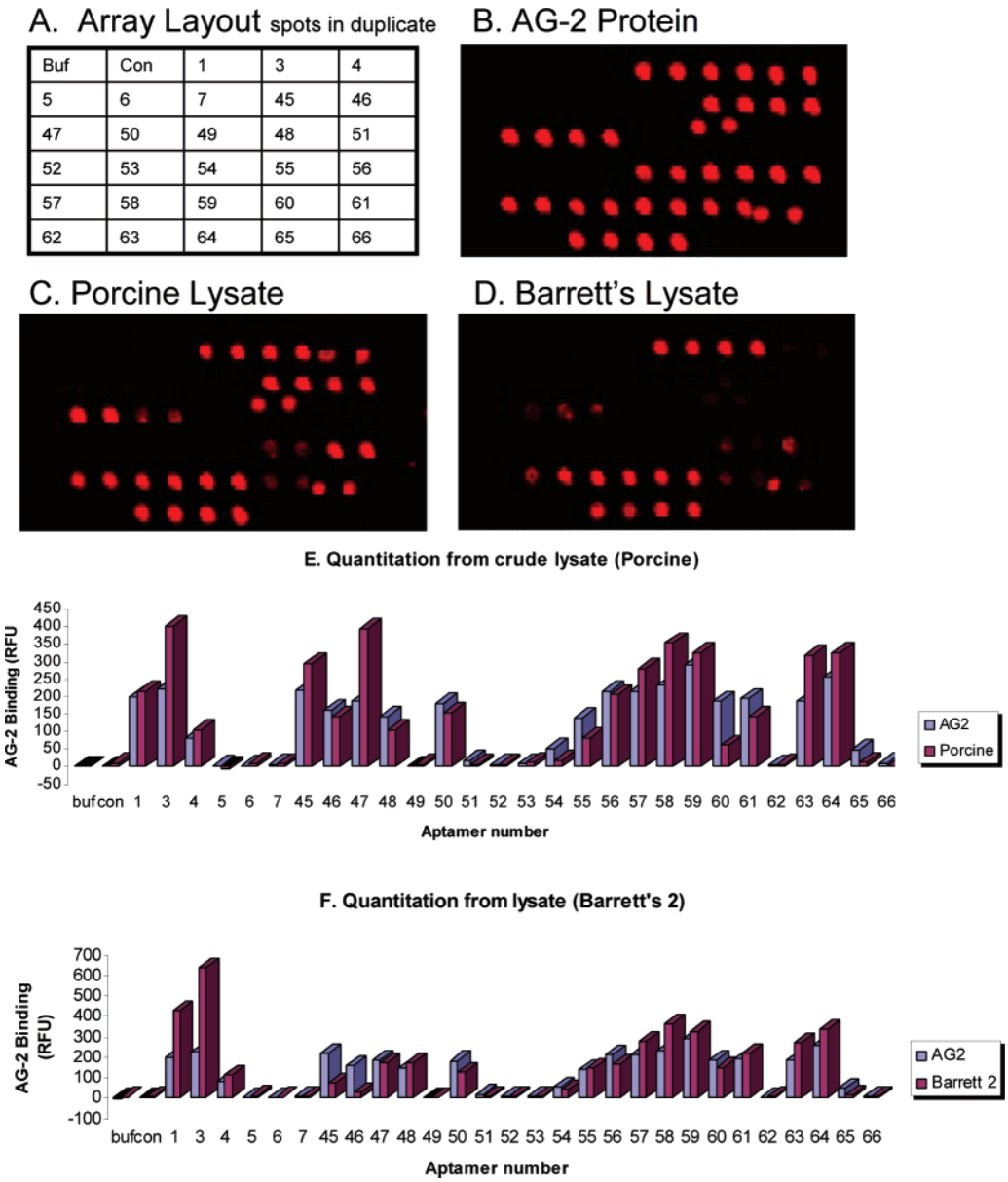
FIGURE 7: Quantitation of recombinant anterior gradient-2 using an aptamer array matrix with fluorescence-based detection. (A) Microarray matrix: Peptide aptamers isolated from the primary screen (the round 2−4 peptides from Figure 1 are listed as A1−D12) were adsorbed in duplicate onto the streptavidin-coated array matrix (5 × 10) as indicated in the experimental procedures. Reactions containing (B) no anterior gradient-2 protein or (C) 0.13 μg/mL anterior gradient-2 protein were incubated on the aptamer array, and the amount of anterior gradient-2 protein bound was quantified in a sandwich assay with anti-rabbit AlexaFluor647conjugate via rabbit anti-AG-2 polyclonal serum. Red dots in the images indicate strong fluorescent response of the aptamer spots which bound AG-2.

lysate when passed over the A4 aptamer column (Figure 6B, lane 5 vs lane 4, arrow) with highly selective enrichment of anterior gradient-2 protein after the low-pH elution (Figure 6B, lane 6 vs lane 4, arrow). This protein was confirmed as being human anterior gradient-2 using mass spectrometry (data not shown). Together, these data establish that the A4 peptide aptamer acquired using recombinant human anterior gradient-2 protein could be used to affinity purify the human protein from a small pinch biopsy.

Microarray-Based Clinical Biomarker Assay Development. To further validate the utility of the aptamer-based capture of a biomarker, we set up quantitative microarray assays using biotinylated peptide aptamers attached to a commercial streptavidin-coated surface in 1 nL spots. Duplicate versions of the aptamers from rounds 2−4 (Figure 1 and listed in Figure 7 as A1−D12) were spotted in duplicate onto the array matrix (Figure 7A) and incubated with a fixed amount of anterior gradient-2 protein for measuring binding capacity. Typical raw data are depicted in Figure 7C vs Figure 7B, where the binding was observed to the key bioactive aptamer (highlighted in Figure 7A, peptides labeled C10, D4, D8, and D11, all of which have the PTTIYY core). Further, dose-dependent quantification and linearity in the detection of anterior gradient-2 protein binding could be detected between 0.03 and 0.5 μg/mL (data not shown). Using this calibration, the capture of anterior gradient-2 protein from crude tissue lysate was measured using the array matrix (laid out in duplicate as in Figure 8A with the alanine substituted and truncated peptides as listed in Figure 4): typically the aptamer series gave rise to the same bioactivity toward anterior gradient-2 protein whether using the pure protein (Figure 8B), the protein in crude porcine lysate (Figure 8C), or the protein from Barrett's epithelial biopsies (Figure 8D). Reductions in the binding of anterior gradient-2 protein from the human biopsy to some peptide variants such as peptides 45 and 46 (Figure 8D), compared to recombinant anterior gradient-2 protein (Figure 8B), are summarized in Figure 8F. This might be due to binding of anterior gradient-2 protein−protein complexes from endogenous tissue that alter the affinity of the anterior gradient-2 protein to the mutated aptamers. This highlights that diagnostic developments using this microarray matrix might need modifications with buffers that expose available epitopes from a target protein to reduce the likelihood of false negatives.

DISCUSSION

Clinical proteomics is a postgenomic approach widely held to be required to provide physiologically relevant data on mechanisms of disease progression and biomarker assay development. The approach is limited by (i) the relative paucity of clinical samples required to extract proteins for target discovery and (ii) the accuracy and speed with which the proteins identified can be validated immunochemically as being actually expressed in clinical material. The generation of polyclonal or monoclonal antibodies to novel proteins identified requires considerable time and effort in terms of antigen preparation, immunization, IgG characterization, and purification prior to validation. A combinatorial technique, such as that commonly used in microbe-based genetic systems, seems the most logical way to advance in a timely manner a high-throughput proteomics validation from clinical samples. Although naive antibody-combining single-chain Fv libraries from native spleen cells could be used as a much more rapid technology to acquire antibody reagents than using classic in vivo immunization methodologies (*20*), the approach is limited by the lower availability of scFv antibody



Buf	Con	1	3	4
5	6	7	45	46
47	50	49	48	51
52	53	54	55	56
57	58	59	60	61
62	63	64	65	66

FIGURE 8: Anterior gradient-2 protein can be quantified from clinical biopsies using an aptamer array matrix. (A) Alanine-substituted and truncated peptide aptamers (numbers listed (1−7 and 45−66) represent peptides as indicated and used in Figure 4) were adsorbed in duplicate onto the streptavidin-coated array matrix (5 × 6) as indicated in the Experimental Procedures. Different amounts of antigen or crude lysate were added to the arrays as probes: (B) purified anterior gradient-2 protein, (C) porcine stomach lysate, or (D) Barrett's biopsy lysate. Raw data from all the spots on the array were converted into relative fluorescent units for (E) recombinant AG-2 protein vs AG2 from porcine stomach lysate or (F) recombinant AG-2 protein vs AG2 from Barrett's lysate.

libraries and the relatively complicated PCR-cloning-based technique that is used to clone and combine the combining sites into a fused single-polypeptide chain (*21*). Optimizations of the scFv by mutagenesis also complicate the applicability of this as a high-throughout approach. By contrast, peptide aptamer sequences identified through phage screening can be rapidly generated synthetically and used in the biotinylated format described in this paper. Further, given that many protein−protein interactions are driven by small linear interaction motifs (*5*), it is possible in theory that peptide combinatorial libraries might be used as a source of a rapidly acquired ligand that can complement antibodies in having a high affinity and specificity for its target. However, the utility of small peptides as affinity, diagnostic, or drug reagents is not very well acknowledged in the cancer field, although the biotechnology sector has exploited these properties to affinity purify a target protein that is clinically relevant (*27*, *28*). Rather, most efforts at developing diagnostic and affinity reagents in basic cancer research have focused on classic monoclonal antibody technologies.

Previous experience in using peptide−phage combinatorial aptamers provided early hope that an in vitro approach to rapidly acquire a high-affinity ligand capable of validating the expression of a clinically relevant target in a biological sample would be possible. Using highly purified recombinant proteins with known protein binding partners, coupled to phage−peptide aptamer libraries, have allowed us to identify novel and specific protein−protein contacts in the established MDM2-p53 and p300-p53 heterocomplexes. This technique has relied on identifying a consensus homology of peptide aptamers acquired through selection to polypeptide motifs within the target protein. For example, MDM2 binding

peptides gave rise to a novel docking site at the *BOX-V* motif in the DNA-binding domain of p53 (*22*), thus identifying a ubiquitination tag that drives the E3 ligase activity of MDM2 (*23*). Further, using p300, proline-repeat-binding peptides were acquired that permitted the identification of a consensus proline-repeat:p300 contact domain that drives DNA-dependent acetylation of p53 (*24*). Finally, identifying a novel p53-binding domain in p300, peptide aptamers were used to demonstrate an intrinsic affinity for polypeptides with homology to the LxxLL-type activation domain of p53 (*25*). Having established this technique as a way to identify novel protein−protein contacts in proteins known to bind to each other, we extended this to the oncogene MDM2 to determine whether novel peptide-binding interfaces could be identified; HSP90 was pulled out and validated as an MDM2-binding protein that could cochaperone with MDM2 and alter the folding of the p53 tetramer (*7*). Thus, a range of high-affinity peptide ligands can be acquired rapidly to a target protein and can be used as bioactive ligands.

With this prior experience in defining linear interaction motifs, we aimed to target a novel, clinically relevant protein identified from a clinical proteomics screen to determine whether peptide aptamers could be acquired, relatively rapidly, with high affinity and most importantly high specificity for a protein target in a crude lysate. Anterior gradient-2 protein was chosen as it was found overexpressed in human disease using proteomics approaches, it can inhibit the tumor suppressor protein p53 (*3*), it provides a novel proto-oncogenic biomarker target in the early detection of esophageal disease (*3*), and it can stimulate metastasis in animal models (*18*). Using recombinant human anterior gradient-2 protein expressed in bacteria, two types of high-affinity peptide aptamers were acquired that can bind anterior gradient-2 protein in an immunochemical blot or ELISA format. The two peptide aptamers bind to two distinct epitopes on anterior gradient-2 protein, on the basis of the observation that one epitope is cleaved by trypsin and the second aptamer binds to a smaller molecular mass polypeptide fragment after cleavage by trypsin. Focusing on one of the highest affinity aptamers as a model, we were able to affinity purify anterior gradient-2 protein from lysates derived from a single clinical biopsy to near homogeneity. These data highlight the ability of peptide aptamers to be isolated toward a clinical target that allows specific capture from a crude material. By comparison, we have attempted during this same time frame to develop monoclonal antibodies toward anterior gradient-2 protein through three immunization−fusion regimes and have still not acquired one monoclonal antibody that can be used for similar affinity purification or diagnostic utility (data not shown). Thus, the specificity and speed of peptide library screening can at least complement the standard monoclonal production route to acquire a reproducible high-affinity ligand toward a target protein.

In summary, peptide aptamers isolated from combinatorial libraries can be used to define novel protein−protein interfaces (*7*, *24*), but also in acquiring a high-affinity ligand that can be used to quantify a clinically relevant target in a crude biopsy lysate. The relatively rapid growth of bacteriophage−peptide particles in selecting for binding ligands coupled to synthetic production of aptamers should increase the efficiency of validating the expression of a protein identified using MS-based technologies from relatively rare clinical material. Current research is now geared toward developing a higher throughput use of the aptamer libraries to a large number of clinically relevant proteins overexpressed in cancers to validate aptamer-captured proteins using mass spectrometry.

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