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Immobilized Peptides as High-Affinity Capture Agents for Self-Associating Proteins

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Summary

There is currently great interest in the fabrication of protein-detecting arrays comprised of large numbers of immobilized protein capture agents. While most efforts in this arena have focused on the use of biomolecules such as antibodies and nucleic acid aptamers as capture agents, synthetic species have many potential advantages. However, synthetic molecules isolated from combinatorial libraries generally do not bind target proteins with the high affinity necessary for array applications. Here, we demonstrate that simple linear peptides bind dimeric proteins tenaciously when immobilized, although they exhibit only modest affinity in solution. These data show that high-affinity bidentate capture agents for dimeric proteins can be created by simply immobilizing modest-affinity ligands on a surface at high density, bypassing the requirement for careful optimization of linker length and geometry that is normally required to create a high-affinity solution bidentate ligand.

Introduction

There is great interest in the development of techniques with which to monitor the levels, posttranslational modification states, and activities of large numbers of proteins simultaneously. One approach is to construct protein-detecting arrays [1, 2], akin to the DNA microarrays used widely in genomics research. Such devices would be comprised of many different protein binding molecules (also called ligands or capture agents) arrayed on a suitable surface in a defined pattern or on coded beads [3, 4], each capable of recognizing its target protein with high affinity and specificity. A significant challenge in the development of such technology will be the isolation of large numbers of suitable protein binding compounds.

Most of the effort in this area has focused on the use of macromolecular biomolecules as capture agents, including antibodies [5–11], nucleic acid (particularly RNA) aptamers [12–16, 41], and protein aptamers [17–19]. However, there are many reasons to explore synthetic capture agents. Synthetic molecules will be easier to produce in large quantities with efficient quality control and can be tailored to allow attachment to surfaces in a defined manner. Also, macromolecules can lose their folded structure and, hence, their activity at surface-solution interfaces, whereas this is not an issue with small molecules. Finally, some types of protein binding synthetic molecules can be produced in bulk and purified far more economically than antibodies or aptamers.

Protein binding molecules can be isolated readily from combinatorial libraries or other types of large compound collections using a number of methods. Unfortunately, small molecules, peptides, peptidomimetics, and other synthetically accessible compounds rarely bind to their target protein with an affinity comparable to that of a good antibody (equilibrium dissociation constant [K_D] $\leq$ 10^{-9} M). Instead, small molecule-protein complexes generally exhibit K_Ds in the μM range, with the exception of molecules optimized through extensive medicinal chemistry efforts or natural selection. This modest affinity is sufficient for some applications, such as chemical genetics studies [20, 21], but is insufficient to capture low abundance proteins from complex mixtures. In addition, the relatively rapid dissociation rates of such complexes result in the loss of most of the bound protein during the inevitable washing steps required to minimize nonspecific "background" binding of high abundance or "sticky" proteins. Therefore, a central problem in applying organic chemistry to the development of protein-detecting microarrays will be to obtain much higher affinity synthetic ligands in a high-throughput fashion.

One potential shortcut in the path from low- to high-affinity binding agents is to create multivalent ligands. Coupling two or more modest-affinity protein ligands with an appropriate linker can provide a high-affinity multidentate capture agent [22–29]. Unfortunately, linker optimization can be time consuming, and most approaches to this problem are unsuitable for high-throughput proteomics applications (but see [30] for an interesting combinatorial approach). However, it seems likely that the issue of linker optimization might be far less important in the case of capturing proteins with immobilized ligands because the surface itself would act as a sort of "combinatorial library of linkers". In other words, if one were to affix two protein binding ligands on a surface in a random fashion and at high density, some fraction of the pairs of immobilized molecules would be oriented appropriately for bidentate binding. Here, we test this idea in the context of a simple model system in which immobilized peptides are examined as capture agents for homodimeric proteins. In this case, there is only one type of ligand on the surface, but two identical molecules must collaborate to bind the target dimer tightly (Figure 1). We show here that, indeed, simple peptides that exhibit modest affinities for their target proteins in solution (K_Ds in the μM range) capture dimeric proteins efficiently when immobilized on a surface. The immobilized peptide-protein complexes are shown to be long lived, with half-lives of hours. Hundreds of human proteins are homodimers or higher-order homooligomers, so this simple approach could provide a large

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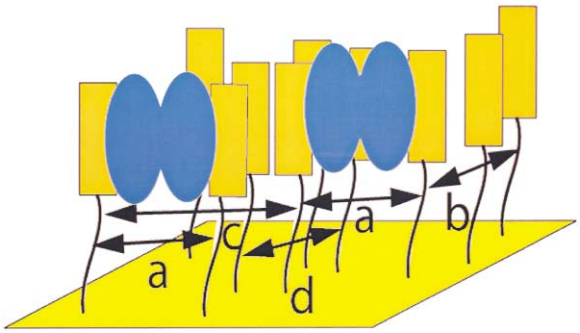

Figure 1. Schematic Diagram of the Anticipated Mode of Binding of a Dimeric Protein with a Surface-Bound Capture Agent

Some fraction of the molecule pairs on the surface are anticipated to have the correct spacing to facilitate high affinity, bidentate binding, as represented by length a. Distances between molecules other than length a, denoted as b, c, and d, will not support high-affinity bidentate binding.

number of capture agents of real utility in the construction of arrays.

Results

Complexes of Immobilized Peptides and Dimeric Proteins Have Long Half-Lives

Peptide libraries are rich sources of protein binding molecules, and there exist many straightforward methods to screen them. As mentioned in the Introduction, most such screens result in the isolation of ligands that form protein complexes with K_Ds in the μM range. To test the surface-mediated avidity concept, we assembled a collection of peptides known to bind different proteins, some homodimers [32, 40] and some monomers [35]. Table 1 presents a list of equilibrium dissociation constants for the complexes used in this study. These values were determined by titrating a low level of fluorescein-labeled peptide with increasing amounts of its protein partner and monitoring the level of binding by fluorescence polarization spectroscopy [31] (see Supplemental Data). The values range from 0.3 μM for the Gal80bp-Gal80 protein complex [32] to 8 μM for the KIXbp1-KIX protein complex. To determine the rate of dissociation of the peptide-protein complexes in solution, a large excess of unlabeled peptide was added to the protein-fluorescent peptide complex, and the time-dependent decrease in polarization of the fluorescence was monitored. As expected from the modest equilibrium dissociation constants, the half-lives of all of these complexes were shorter than the time required to mix the solutions, which was about 10 s (data not shown).

With these solution values in hand, the binding properties of the same peptides immobilized on Tentagel beads were evaluated. Each peptide was synthesized on Tentagel resin modified with an acid-stable linker, allowing deprotection of the peptide side chains without severing the link to the bead. Figure 2A shows the protocol that was employed to evaluate the kinetic stability of the immobilized peptide-protein complexes. The complex was formed by addition of excess protein to 18 mg of peptide-coated beads. After washing, the beads were divided equally into nine tubes and then added to a large volume of buffer (10 or 15 ml), such that if the bound protein dissociated from the bead, it would presumably be unable to reassociate due to its high dilution. To further inhibit reassociation, the buffer was also changed every 15 min to remove any free protein. The amount of protein remaining on the beads was monitored at 15 min intervals by denaturing gel electrophoresis and Western blotting.

Figure 2B shows the results of experiments that employed the Gal80, GST-Pre1, and VEGF proteins, all native dimers, as well as dimeric fusions of Mdm2 (GST-Mdm2) and the KIX domain of CREB binding protein (GST-KIX). In the case of Gal80 protein, the level of bound protein decreased to approximately 50% of the original level within 30 min, after which the level of retained protein remained constant for at least an additional 1.5 hr. This biphasic behavior was expected, based on the fact that the stable Gal80 dimers associate to form tetramers with a K_D in the mid to high nM range [33, 34]. Since the beads were originally exposed to excess Gal80 at a protein concentration above the K_D of the tetramer, it seems likely that this form of the protein was the dominant species bound, but that only one of the component dimers was in contact with the immobilized peptides. Thus, we interpret these results as an initial dissociation of the Gal80 tetramer, leaving

Table 1. Peptides Employed in This Study and the Equilibrium Dissociation Constants (K_D) of the Peptide-Protein Complexes

Name	Reference	Sequence	K_D (μM)
Gal80bp	32	YDQDMQNNTFDDLFWKEGHR	0.3
Gal80bpscram		DLQRDTNKGFHEMFDWDYQN	ND
Pre1bp	This work	**SHS**TARGEQERAAVYLWFTYDHR**SER**	[a]
Pre1bpscram		SEFARDLAYGEYSQHVRWTHERATSR	ND
VEGFbp	40	RGWVEICAADDYGRCLTEAQ	>1[b]
VEGFbpscram		CQECDYWREVRGADALITGA	ND
Mdm2bp	35	PLSQETFSDLWKLLPENNV	2
Mdm2bpscram		NVKWLDPNQELPSFLTSLE	ND
KIXbp1	This work	SVPGSVSWFEFWSAVDAVET	8
KIXbp1scram		FSASFTEVVDAGWVSPWSVE	ND

The peptide names indicate the protein they recognize. Abbreviations: bp, binding peptide; ND, not determined; scram, scrambled. The residues shown in bold in the Pre1bp were added to enhance solubility. All peptide sequences are written with the N-terminus at the left end.

[a] Could not be determined due to limited solubility of the peptide.

[b] Lower limit. The true value could not be measured due to a limited amount of protein.

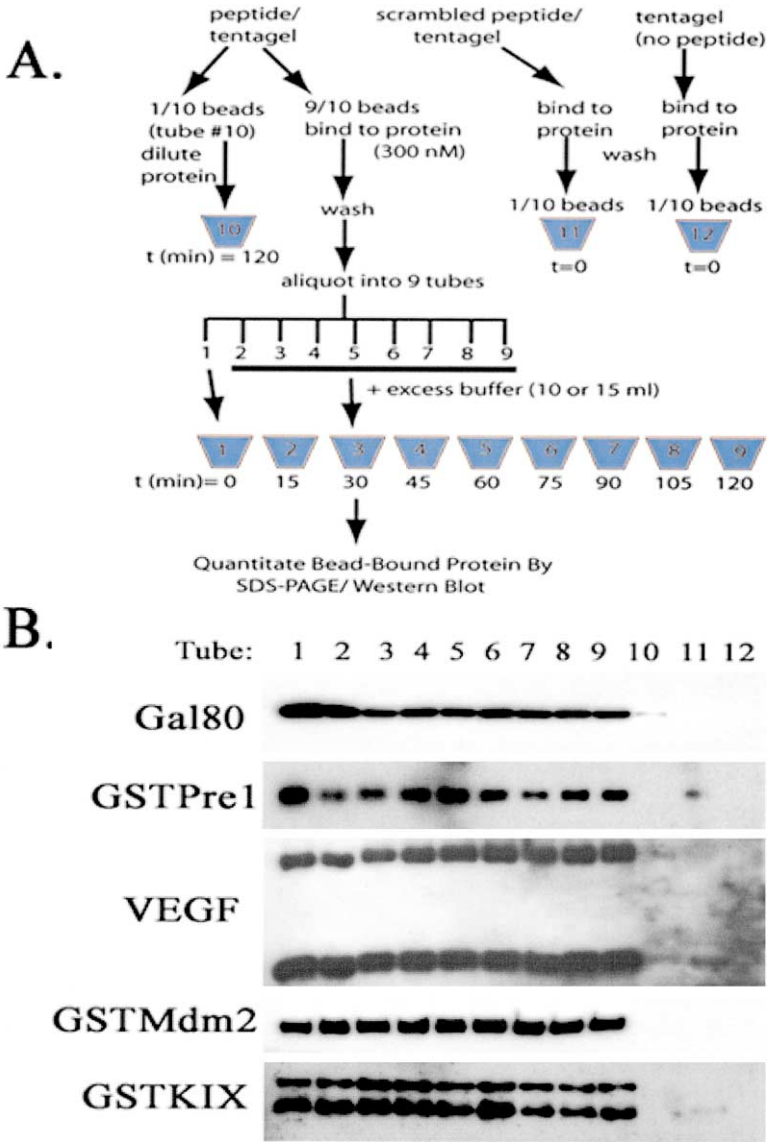

Figure 2. Slow Dissociation of Proteins from Tentagel-Bound Binding Peptides

(A) Schematic representation of the assay employed to monitor the half-lives of the immobilized peptide-protein complexes.

(B) SDS-PAGE/Western blot analysis of the amount of each protein remaining on the peptide-coated beads following dilution. Time elapsed following dilution is indicated in (A) for each tube. Lane 10 contains samples in which the peptide-coated beads were added to the highly dilute protein solutions (≈5 pmol) and incubated for 2 hr to control for protein reassociation with the beads under these conditions. Lane 11 represents experiments using scrambled sequences attached to the beads. Lane 12 represents experiments done with Tentagel beads lacking any peptide. Two bands are observed in the VEGF experiment because the homodimer is linked by disulfide bonds that were incompletely reduced prior to electrophoresis.

behind a tightly bound Gal80 dimer that does not dissociate from the beads during the course of the experiment. GST-Pre1, VEGF, GST-KIX, and GST-Mdm2 were also bound stably to the beads. Little if any dissociation of these dimeric proteins was observed over the course of 2 hr. To ensure that these results truly represented stable, specific peptide-protein complexes, several controls were done. Lane 10 in Figure 2B shows the result of adding an amount of protein equal to the "t = 0" level to the highly dilute bead plus buffer mixture and incubating for 2 hr. In accordance with the procedure mentioned above, the buffer was exchange every 15 min. This control was done to assess whether the protein could reassociate with the beads under the conditions of the kinetic lifetime experiment. No association of the VEGF, GST-Pre1, GST-KIX, or GST-Mdm2 was observed under these conditions, and only a trace of Gal80 was present. The experiment was also repeated with beads displaying a scrambled version of the binding peptide (see Table 1) and beads lacking any peptide. In each case, no bound protein was observed, with the exception of a small amount of binding of the Pre1 protein to the scrambled peptide. This presumably represents a minor nonspecific interaction. We conclude from this experiment that the half-lives of these immobilized peptide-protein complexes are several hours or more, an increase of at least three orders of magnitude compared to that measured in solution (>2 hr as compared to <10 s).

Comparison of the Kinetic Stability of Complexes of Immobilized Peptides with Analogous Monomeric and Dimeric Proteins

It seems likely that the huge differences between the kinetic stabilities of these peptide-protein complexes in solution and on beads is due to bidentate binding of the immobilized peptides to the dimeric proteins (Figure 1). However, other possibilities cannot be ruled out based on these data alone. To probe this issue further,

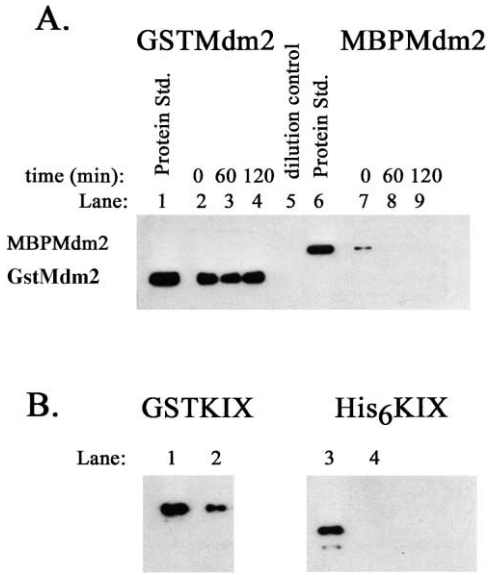

Figure 3. Comparison of the Dissociation Rates from Immobilized Peptides of Monomeric and Dimeric Fusion Constructs Containing the Same Target Protein

A protocol identical to that shown in Figure 2A was employed, except fewer time points were taken.

(A) SDS-PAGE/Western blot analysis showing the levels of dimeric GST-Mdm2 or monomeric MBP-Mdm2 remaining associated with Tentagel-Mdm2bp after dilution for the time indicated.

(B) A similar experiment was conducted with dimeric GST-KIX or monomeric His6-KIX. Lanes 1 and 3 show the protein standards; lanes 2 and 4 show the amount of protein remaining on the Tentagel-KIXbp1 beads immediately following the washing step.

experiments were carried out using dimeric and monomeric versions of the same protein.

The domain of the human Mdm2 protein represented by residues 1–188 is a structurally characterized monomer. This Mdm2 fragment is known to bind a peptide derived from the p53 activation domain [35]. Two different Mdm2-containing fusion proteins were expressed and purified. In one, the Mdm2 domain was fused to maltose binding protein (MBP), a monomer. In the other, it was fused to GST, a native dimer. Titration experiments showed that in solution the p53-derived peptide bound each form of the protein with similar affinity and kinetics ($K_D \approx 2$ μM [Table 1] and with a half-life of less than 10 s). The p53-derived peptide had no detectable affinity for GST or MBP alone (see Supplemental Data).

The kinetic stabilities of each of these complexes were then probed when the peptide was immobilized on Tentagel beads using a dilution protocol similar to that shown in Figure 2A, except that fewer time points were taken. As shown in Figure 3A (lanes 6–9), monomeric MBP-Mdm2 dissociated from the beads rapidly. Only a small fraction of the input protein was detectable immediately after completion of the washing steps (Figure 3A, lane 7), and no trace of protein was detectable on the beads after a 1 hr incubation (lane 8). In stark contrast, little or no dissociation of the dimeric GST-Mdm2 fusion protein was observed even after 2 hr. Again, a dilution control demonstrated that if the protein had dissociated from the beads under these conditions, reassociation would not have occurred.

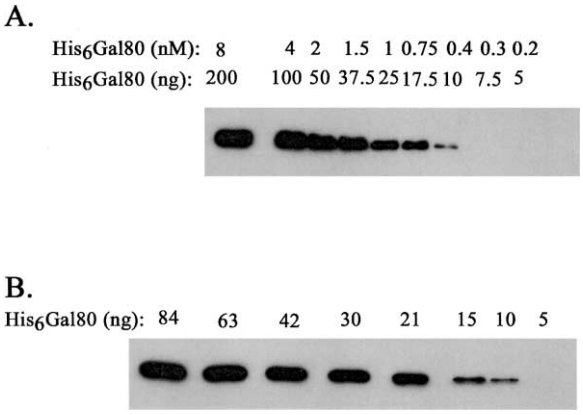

Figure 4. Efficient Capture of a Dilute Protein by Immobilized Peptides

(A) The amount of His6Gal80 protein indicated was added to 500 μl of buffer containing 5.7 mg *E. coli* lysate and 2 mg of Tentagel-Gal80bp. After incubation and washing, the amount of protein retained on the beads is shown.

(B) Calibration blot using known amounts of purified His6Gal80 protein.

Similar experiments were also carried out with monomeric and dimeric forms of the KIX domain [36]. In this case, the peptide employed was isolated by phage display from a library of 20-mers (see Experimental Procedures). The peptide-protein complexes (including either monomeric His_6-KIX or dimeric GST-KIX) exhibited K_Ds of approximately 8 μM in solution (Table 1). However, the half-lives of the two peptide-protein complexes differed greatly when the peptide was immobilized on Tentagel. Figure 3B (lanes 2 and 4) displays the amount of protein remaining on the beads immediately after the washing steps. Monomeric His_6-KIX was undetectable, whereas dimeric GST-KIX was present. As was shown in Figure 2, GST-KIX bound to immobilized KIXbp1 possessed a half-life in excess of 2 hr.

The striking differences between the half-lives of complexes containing monomeric and dimeric forms of the same protein on the peptide-coated Tentagel beads strongly supports the idea that stable binding of native dimers is due to bidentate binding.

Efficient Capture of a Dilute Protein

In all of the above experiments, the protein was loaded onto the peptide-coated beads at a relatively high concentration (300 nM) prior to dilution. Since the concentration of most proteins of interest in a biological sample will be lower, it was of interest to evaluate the ability of a Tentagel-bound peptide to capture a dimeric protein from more dilute solutions. Figure 4 shows the results of an experiment in which 2 mg of Gal80bp-coated Tentagel beads (≈0.4 μmol of peptide) was incubated with the indicated amounts of Gal80 protein plus 5.7 mg of *E. coli* lysate in a volume of 500 μl. After washing, the bound protein was detected by boiling the beads in denaturing loading buffer followed by SDS-PAGE/Western blot analysis. Figure 4B shows a Western blot in which known amounts of purified Gal80 were applied to the gel, allowing quantitation of the amount of protein

retained by the bead-bound peptide in the experiment. The results show that even at the lowest protein concentration detectable in this assay (0.4 nM), essentially 100% of the protein was bound to the beads, as can be seen by comparing the band intensities in the experimental and calibration blots (Figure 4). At 0.4 nM, Gal80 represented only 0.16% of the total protein concentration, so this experiment is a reasonable model for capture of a protein of modest abundance from a crude extract. Further dilution of the protein in this assay format exceeded the sensitivity of the Western blot. This suggests that the effective K_D of the immobilized peptide-Gal80 complex must be at least an order of magnitude lower than 0.4 nM.

Specificity of the Peptide-Protein Interaction

In a protein-detecting array, a complex solution such as a blood sample or cell extract containing thousands of proteins would be applied to the array. For proper interpretation of the results, it is critical that the specificity of binding of the target protein to its cognate capture agent is high.

The data shown in Figure 4, an experiment that employed low levels of Gal80 doped into a crude bacterial extract, suggest that the peptide binds quite specifically. But to address this point further, the composition of proteins retained by Tentagel-bound Gal80bp was assessed directly. His_6Gal80 was doped into an *E. coli* lysate such that Gal80 represented 5% of the total protein concentration. This solution was then incubated with the Tentagel-bound Gal80bp followed by thorough washing to remove any unbound material. The composition of the proteins captured by the peptide was then addressed by SDS-PAGE followed by silver staining. As shown in Figure 5, Gal80 was the only protein retained from the extract, at least at the level detectable by silver staining (compare lane 4 to the input, lane 2). No detectable proteins were retained in a control experiment using a scrambled version of Gal80bp (Figure 5, lane 5). To provide context for this result, the same Gal80-doped extract was applied to glutathione-sepharose-bound GST-Gal4 AD. This 34-residue fragment of Gal4 is the native ligand for Gal80 and is known to form a high-affinity and -specificity complex with the repressor [37]. As can be seen by comparing lanes 4 and 6 in Figure 5, the results obtained using the peptide and the native Gal4 AD were quite similar (the intense doublet of bands near the bottom of the gel in lanes 6 and 7 represent the GST-Gal4 AD fusion protein that was eluted from the beads).

Fluorescence-Based Detection of Captured Protein

All of the experiments described above have employed Western blotting to detect the captured protein. Of course, in a true microarray experiment this would be impossible. More commonly, captured proteins are detected via fluorescence methods either because they have been labeled directly or through a sandwich assay using a fluorescently labeled antibody. To demonstrate that peptide-based avidity capture elements work in this format, the experiment shown in Figure 6 was conducted. Tentagel beads displaying the Mdm2 binding peptide were incubated with Texas red-labeled GST-Mdm2 protein (at concentrations ranging from 100 nM to 1 nM.) The beads were then washed rigorously and photographed. As shown in Figure 6, the fluorescence signal due to the captured GST-Mdm2 protein was readily visible and increased in intensity with increasing protein concentration.

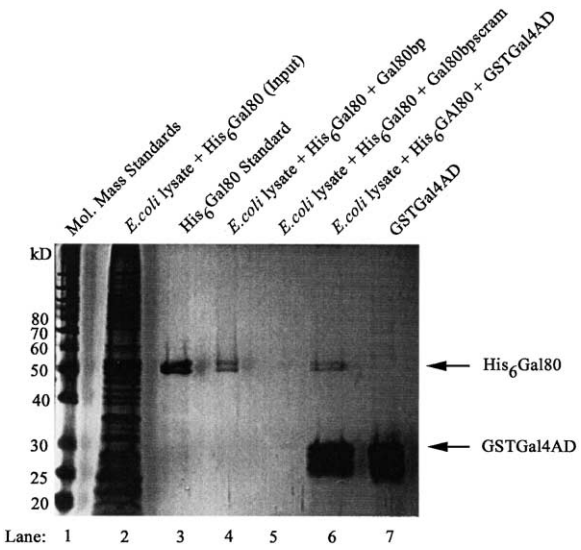

Figure 5. Tentagel-Gal80bp Retains Gal80 Protein Specifically from an Extract

His6Gal80 protein (lane 3) was doped into an *E. coli* extract such that it constituted 5% of the total protein. This solution (lane 2) was incubated with Tentagel-Gal80bp (lane 4), Tentagel-Gal80bpscram (lane 5), or a GST-Gal4 AD fusion protein bound to glutathione-sepharose beads (lane 6). After washing, the proteins retained in each case were analyzed by boiling in denaturing buffer, followed by SDS-PAGE and silver staining. His6Gal80 was the only protein detectable in the Tentagel-Gal80bp-bound fraction (lane 4), and the specificity of binding appeared comparable to that shown by the Gal4 activation domain, Gal80's native ligand (compare lanes 4 and 6).

Discussion

The creation of high-affinity protein ligands via the linkage of two or more lower-affinity binders is well documented. However, the application of this general concept to proteomics, which will demand high throughput, has been hampered by the fact that high affinity is realized only with a linker that is of appropriate length and geometry. We postulated that for immobilized ligands the issue of linker optimization would be minimized or eliminated for two reasons. First and most importantly, the surface itself would act as a sort of combinatorial library of linkers (Figure 1), allowing some fraction of the pairs of ligands to act as high-affinity receptor sites for dimeric proteins (Figure 1). Second, the advantage of bidentate binding in soluble ligands is always compromised to some degree by the energetic cost of restricting the degrees of freedom of the linker. It seemed likely to us that this cost would be far less in the case of an array surface acting as the linker than for a standard

Figure 6. Capture of Texas Red-Labeled Protein with Immobilized Peptides

Texas red-labeled GST-Mdm2 was exposed at the indicated concentration to Tentagel beads displaying the Mdm2 binding peptide. After washing, the beads were photographed.

solution linker. The seminal result of this report is that these expectations appear to have been realized. Peptide ligands with modest affinities for their target dimeric proteins in solution serve as very high-affinity ligands when immobilized on beads. However, no such large difference was observed when the target protein was a monomer.

We chose Fmoc(aminoethyl)-Photolinker NovaSyn TentaGel resin (Nova Biochem) as a model support, since the photocleavable linker is acid stable. This allows for the deprotection of the side chains without releasing the peptide and alleviates the need to couple the peptide to a solid surface postsynthesis. We emphasize that Tentagel beads are unlikely to be the support actually employed to construct protein capture arrays, but they were convenient and suitable for this initial study of avidity effects. Other surfaces, such as chemically modified glass slides, can also provide high densities of ligands [42].

Using the Tentagel-bound, 20-residue Gal80 binding peptide [32] as a model system, the following observations were made. Gal80 protein was retained almost quantitatively from a dilute (0.4 nM) solution containing a large excess of bacterial proteins (Figure 4). The immobilized peptide-protein complex exhibited a half-life of well over 2 hr (Figure 2). The peptide exhibited excellent specificity, as evidenced by the fact that Gal80 was the only protein retained by the peptide when it was mixed with a Gal80-doped *E. coli* extract (Figure 5). Stable binding of immobilized peptides to dimeric proteins was also observed using peptides that bind Pre1, VEGF, and the dimeric GST fusions of Mdm2 and the KIX domain. However, monomeric proteins containing the KIX domain and Mdm2 bound to their cognate immobilized peptides much more weakly.

The high kinetic and thermodynamic stability of the immobilized peptide-homodimeric protein complexes studied here argues that practically useful protein-detecting arrays could be constructed using these simple synthetic molecules as capture agents. Of course, this strategy will be limited to the special case of homodimeric or higher-order proteins. Nonetheless, a search for human homodimers, homotrimers, or homotetramers using the protein information resource database sponsored by Georgetown (http://pir.georgetown.edu) resulted in 154 known examples. It stands to reason that the true number of self-associating proteins is much higher, since only a fraction of the human proteome has been studied at this level of detail. Thus, it seems reasonable to suggest that several hundred proteins could be "covered" in a protein-detecting array using simple peptides as capture agents. Of course, the same principle of bidentate binding should hold true for non-peptidic molecules isolated by screening synthetic libraries. We note that there exist methods to screen cDNA libraries for genes that encode self-associating proteins [38], so it should be possible to compile a list of proteins for which this simple approach would be applicable.

Significance

There is great interest in the construction of protein-detecting microarrays. One of the biggest challenges in the development of such devices will be the isolation of large numbers of protein capture agents. We demonstrate here that for the special case of self-associating proteins, simple linear peptides can be employed as efficient protein capture agents due to bidentate contacts between the immobilized ligands and the dimeric protein. This suggests that relatively complex protein-detecting microarrays could be constructed more easily and rapidly than imagined previously.

Experimental Procedures

Plasmids

pQE60/PRE1 and pProEX-1/Gal80, the expression vectors for His_6PRE and His_6Gal80, were provided by Prof. Stephen Johnston (UT Southwestern). pQE60 places the His_6 tag at the carboxyl terminus of PRE1, while the His_6 tag for pProEX-1/Gal80 is at the N terminus of Gal80. The GST fusion of PRE1 was constructed by inserting a PCR amplicon containing the *PRE1* gene into Nco1/BamH1-cut pGEXCSTEV plasmid (provided by Prof. Johnston). The plasmid expressing GST-fused human Mdm2, pGEX Mdm2, was provided by Prof. David Lane, University of Dundee. Construction of pMal-c2X-Mdm2 was achieved by restriction digest of pGEX-Mdm2 with BamHI/EcoRI and ligation of the resulting fragment into the pMAl-c2X plasmid purchased from NEB. The plasmid encoding full-length mouse CREB binding protein (CBP) was pRC/RSV-mCBP-HA-RK. A fragment of CBP including the KIX domain, aa 378–817, was amplified by PCR and inserted into BamHI/HindIII-digested pRSET-A vector (Invitrogen) to produce the resultant plasmid pRSET-CBP(378–817), which expresses the His_6 tag at the N terminus of the protein. The plasmid expressing the GST fusion of CBP(378–817), pGEX-2T-CBP(378–817), was constructed by insertion of the same CBP amplicon into BamHI/HindIII-digested pGEX-

2T vector purchased from Pharmacia Biotech that was engineered with a HindIII site. A His_6 tag was added to the pGEX 2T-CBP (378–817) construct at the carboxyl terminus of CBP by amplifying the CBP fragment with the primers 5′-CC GCG GGA TCC GCC TGT TCT CTC CCA CAC TGT CG-3′ and 5′-GAA TTC AAG CCT TTA GTG GTG ATG GTG GTG ATG GGC TGC TGG TTG CCC CAT GCC CAC AC-3′ and inserting back into the BamHI/HindII-digested pGEX 2T vector.

Proteins and Antibodies

Glutathione-S-transferase (GST)-fused proteins were expressed in *E. coli* cells grown in LB media plus ampicillin to an OD_{600} of 0.6–0.8 and induced with 1 mM IPTG for 2–3 hr at 37°C. The cells were lysed using sonication and centrifuged at 87,000 × g to remove cell debris. Glutathione sepharose beads (Pharmacia Biotech) equilibrated with PBS buffer were added to the lysate and incubated for 1 hr at 4°C with agitation. The beads were poured into a column and washed with 20 column volumes of PBS plus 0.5% Triton X-100 followed by 10 column volumes of PBS buffer. The GST protein was eluted from the beads with 10 mM reduced glutathione in 50 mM Tris (pH 8.0) and dialyzed into PBS buffer plus 10% glycerol.

His_6-tagged fusions were expressed using the same protocol as described above for the GST fusions. After centrifugation, Triton X-100 was added to 1%. Ni-NTA beads (Qiagen) that had been equilibrated with 50 mM sodium phosphate buffer (PB) + 300 mM NaCl were added to the lysate and incubated for 1 hr at 4°C. The beads were poured into a column, washed with 20 column volumes of PB + 500 mM NaCl + 0.1% Triton X-100 + 40 mM imidazole followed by 10 column volumes of 50 mM sodium phosphate buffer (PB) + 300 mM NaCl. The His_6-tagged protein was eluted from the beads with 400 mM imidazole and dialyzed into PBS buffer + 10% glycerol.

MBP-Mdm2 was expressed in *E. coli* cells grown in rich broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) + glucose (2 g/liter) + ampicillin to an OD_{600} of 0.5 and induced with 0.3 mM IPTG for 2 hr at 37°C. The cells were lysed using sonication and centrifuged at 87,000 × g to remove cell debris. Amylose resin (NEB) equilibrated with column buffer (20 mM Tris + 200 mM NaCl + 1 mM EDTA) was added to the lysate and incubated for 1 hr at 4°C. The beads were poured into a column and washed with 20 column volumes of column buffer. The MBP-Mdm2 was eluted from the beads with column buffer + 10 mM maltose and dialyzed into PBS buffer + 10% glycerol.

All other proteins were obtained from commercial sources including VEGF (USBiological), MBP (NEB), and glutathione-S-transferase (Sigma). Antibodies for Western blotting were provided by Prof. Stephen Johnston, with the exception of mouse anti-GST (sc-138) and goat anti-VEGF (sc-152 G) purchased from Santa Cruz and mouse anti-Penta Histidine purchased from Qiagen.

Synthesis of Peptides

Peptides were synthesized using a Symphony peptide synthesizer (Protein Technology Incorporated) via Fmoc chemistry on Fmoc(aminoethyl)-Photolinker NovaSyn TentaGel resin (Nova Biochem). The linker is resistant to cleavage with TFA; therefore, the peptide side chains can be deprotected with TFA without releasing the peptide from the bead. The bead-bound peptides were sequenced by automated Edman degradation on an Applied Biosystems 476A Protein Sequencer. Comparison of the peptide sequencing peaks to a standard of known concentration indicated that a single Tentagel bead contained approximately 100 pmol of full-length peptide.

Fluorescently labeled peptides were synthesized using Fmoc chemistry on Rink Amide MBHA resin (Nova Biochem). The peptides were modified at the N terminus with 5(6)-carboxyfluorescein (Fluka) activated with HBTU (Advanced Chemtech). Peptides were cleaved from the resin with TFA, and purification was performed on a Biocad Sprint HPLC. The masses of each peptide were analyzed by MALDI-TOF mass spectrometry (Voyager DE Pro, Applied Biosystems) and were within 0.1% of the predicted mass.

Phage Display

A 20-amino-acid peptide library expressed at the N-terminal pIII of M13 phage (named ON.543) was provided by Prof. Stephen Johnston (originally obtained from Affymax [39]) and contained approximately 10^8 different peptides. Pre1 binding peptide (PREbp) was isolated after six rounds of panning as follows. Round (RD) 1: His_6PRE1 (40 μg) was absorbed onto an ELISA plate, incubated with 10^8 phage in PBS (20 mM sodium phosphate [pH 7.5] and 150 mM NaCl) buffer for 2 hr at room temperature, washed 8 times with 1× PBS + 0.1% Tween 20, and eluted with 50 mM glycine HCl (pH 2.0). RD 2: GST Pre1 (80 μg) was bound to glutathione beads, incubated with 10^8 phage from RD1 in PBS + 0.5% Triton X-100 for 2 hr at 4°C, washed 4 times with PBS + 1% Triton X-100, then 2 times with PBS, and eluted by cleavage with TEV protease. RD 3: GST Pre1 (40 μg) was bound to glutathione beads, incubated with 10^9 phage from RD2 in PBS + 1% Triton X-100 for 2 hr at 4°C, washed 4 times with PBS + 1% Triton X-100 + 350 mM NaCl, then 2 times with PBS, and eluted by cleavage with TEV protease. RD 4 was the same as RD 3. RD 5: His_6PRE1 (12 μg) was bound to Ni-NTA beads, incubated with 10^{10} phage from RD 4 in PBS + 1% Triton X-100 + 10 mM imidazole for 2 hr at 4°C, washed 4 times with PBS + 1% Triton X-100 + 350 mM NaCl + 20 mM imidazole, then 2 times with PBS, and eluted with 200 mM imidazole, RD 6 was the same as RD 5. After RD 6, phage DNA was isolated and sequenced. Since the free synthetic peptide was insoluble, an additional six polar amino acids were added, three on each end, to enable the peptide to be displayed on beads.

CBP(378–817) binding peptide (KIXbp1) was found after four rounds of panning as follows. RD 1: GSTCBP(378–817) was absorbed onto an ELISA plate, incubated with 10^8 phage in PBS (20 mM sodium phosphate [pH 7.5] and 150 mM NaCl) buffer for 2 hr at room temperature, washed 8 times with 1× PBS + 0.1% Tween 20, and eluted with 50 mM glycine HCl (pH 2.0). RD 2: GSTCBP(378–817) was bound to glutathione beads, incubated with 10^9 phage from RD1 in PBS + 1% Triton X-100 for 2 hr at 4°C, washed 4 times with PBS + 1% Triton X-100, then 2 times with PBS, and eluted with 20 mM glutathione in 50 mM Tris-HCl (pH 8.0). RD 3 was the same as RD 2. RD 4: GSTCBP(378–817) was bound to glutathione beads, incubated with 10^9 phage from RD3 in PBS + 1% Triton X-100 for 2 hr at 4°C, washed 3 times with PBS + 1% Triton X-100 + 400 mM NaCl, then 2 times with PBS, and eluted with 20 mM glutathione in 50 mM Tris-HCl (pH 8.0). After RD 4, phage DNA was isolated and sequenced.

Determination of Solution Binding Constants

Titration experiments were monitored by fluorescence polarization spectroscopy using a PanVera Beacon 2000 instrument capable of measuring anisotropy of fluorescein-labeled molecules. Approximately 5 nM of the fluorescein-labeled peptide was added to 200 μl of PBS buffer (with the exception of VEGF, where to conserve protein only 100 μl volume was used) which also contained 0.2 mg/ml bovine serum albumin and various concentrations of the target protein. The samples were incubated for 20 min at room temperature to allow equilibrium to be reached. The sample was then placed into the fluorescence spectrometer, and the polarization of the emitted light was measured. A plot of the change in anisotropy versus the protein concentration (see Supplemental Data) was used to determine the dissociation constant (K_D).

To determine the rate of dissociation of the peptide-protein complex in solution, the fluorescein-labeled peptide and the partner protein were incubated at a protein concentration 10-fold above K_D. Unlabled peptide was then added at a concentration 10- to 100-fold above the labeled protein concentration. Fluorescence ansotropy measurements were taken every 30 s. The anisotropy versus time was plotted and fit to a first-order decay equation. In all cases, the dissociation reaction was >90% complete in the time required to mix the unlabeled peptide with the complex and return the cuvette to the spectrometer (approximately 10 s).

Comparing Monomeric and Dimeric Proteins

Approximately 0.02 g of p53 peptide/Tentagel beads was blocked with 10% milk overnight at 4°C. The beads were split into two tubes, and 6 μM of GSTMDM2 or MBPMDM2 was added along with 1% milk. The beads were incubated 2 hr at 4°C and then washed four times at room temperature for 5 min with 10 mM Tris (hydroxymethyl) aminomethane (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 (TBST

buffer), then two times with 20 mM sodium phosphate (pH 7.5) and 150 mM NaCl (PBS buffer). After washing, each sample was aliquoted into three tubes with excess buffer (15 ml PBS) added to tubes 2 and 3 and exchanged every 15 min. The dissociation of the peptide-protein complexes was monitored at 1 hr and 2 hr. Gel loading dye (50 mM Tris base [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue, and 0.1% xylene cynanol) was added directly to the beads, which were then boiled. After cooling, the supernatant was loaded onto a SDS (sodium dodecyl sulfate) polyacrylamide gel and analyzed by Western blotting.

The same procedure as above was carried out for GST-KIX and His_6KIX at a protein concentration of 300 nM, with the exception that BSA (3% for blocking and 0.3% for binding) was used instead of milk.

Dissociation of Protein from Peptide on Tentagel Beads

Approximately 0.02 g of peptide/Tentagel beads was blocked with 10% milk (except PRE1bp was blocked with 3% BSA) overnight at 4°C. One-tenth of the beads were aliquoted and set aside to be used as a control (tube 10). To the remaining beads, target protein (500 μl of a 300 nM solution) was added along with 1% milk (or 3% BSA + 1% Triton X-100 for PRE1) and allowed to bind for 2 hr at 4°C. The beads were then washed four times at room temperature for 5 min with 10 mM Tris (hydroxymethyl) aminomethane (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 (TBST buffer), then two times with 20 mM phosphate (pH 7.5) and 150 mM NaCl (PBS buffer). PRE1bp was washed four times at room temperature for 5 min with 20 mM phosphate (pH 7.5), 350 mM NaCl, and 1% Triton X-100, then two times with PBS buffer. The beads were then equally divided into nine tubes, and 10 or 15 ml of excess buffer was added to tubes 2–9. To control for possible rebinding of dissociated protein, in tube 10 the protein was added to the peptide on beads after the addition of excess buffer. The dissociation of the complex was monitered over a 2 hr period with exchange of buffer every 15 min. Gel loading dye was added directly to the beads, and after boiling the supernatant was loaded onto a SDS polyacrylamide gel and analyzed by Western blotting.

Capturing Dilute Proteins

Approximately 0.018 g of Tentagel beads displaying Gal80 binding peptide was blocked with 10% milk overnight at 4°C. The beads were divided equally into nine tubes and incubated with 500 μl of buffer containing *E. coli* lysate (12 mg/ml) plus various amounts of His_6Gal80 protein (5–200 ng) for 2 hr at 4°C. After washing four times with TBST buffer and twice with PBS, gel loading dye was added directly to the beads, and the amount of bound His_6Gal80 was determined as described above. A second gel containing known amounts of pure His_6Gal80 was analyzed simultaneously and was used as a standard to quantitate the amount of His_6Gal80 retained on the beads.

Specificity of Gal80 Binding

E. coli lysate was prepared by growing BL21 cells to an OD_{600} of 0.8. Cells were lysed by sonication in PBS buffer. After sonication, Triton X-100 was added to 1%. His_6Gal80 was doped into the *E. coli* lysate such that it constituted 5% of the total protein. Approximately 0.02 g of Tentagel beads on which the Gal80 binding peptide had been synthesized and deprotected was added to 300 μl of the *E. coli* lysate containing His_6Gal80 and incubated for 2 hr at 4°C. The beads were washed four times for 10 min each time at 4°C with PBS buffer + 1% Triton X-100 + 150 mM NaCl and then twice with PBS buffer. GSTGal4 AD (activation domain) was preincubated with glutathione sepharose beads and then used as a positive control to pull down His_6Gal80 from the lysate under the same conditions. Gel loading dye was added directly to the beads, loaded onto a SDS polyacrylamide gel, and analyzed by silver stain.

Fluorescence-Based Detection of Captured Protein

GST-Mdm2 protein was labeled with Texas red NHS-ester (Molecular Probes) according to the recommended protocol. Tentagel beads containing the p53-derived peptide were blocked with 2% bovine serum albumin for 1hr at room temperature. Texas red-labeled GST-Mdm2 was added to the beads at various protein concentrations (100, 10,and 1 nM) in TBST buffer containing 2% BSA. After incubating for 1 hr t room temperature, the beads were washed six times with TBST buffer and then visualized using a Nikon Eclipse TE300 fluorescence microscope equipped with a CCD camera. An excitation filter of 570 ± 10 nm was used to illuminate the beads,and an emission filter of 630 ± 30 nm was used to collect the Texas red emission signal. Pictures were taken with an exposure time of 1000 ms, binning of 4, and 10× magnification.

Supplemental Data

A figure showing titration experiments monitored by fluorescence polarization to determine the equilibrium dissociation constants of the peptide-protein complexes employed in this study is available at http://www.chembiol.com/cgi/content/full/10/3/251/DC1 or by writing to chembiol@cell.com for a PDF.

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