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A designed repeat protein as an affinity capture reagent

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Abstract

Repeat proteins are an attractive target for protein engineering and design. We have focused our attention on the design and engineering of one particular class - tetratricopeptide repeat (TPR) proteins. In previous work we have shown that the structure and stability of TPR proteins can be manipulated in a rational fashion [Cortajarena 2011; Main 2003]. Building on those studies, we have designed and characterized a number of different peptide-binding TPR modules and we have also assembled these modules into supramolecular arrays [Cortajarena 2009; Cortajarena 2008; Jackrel 2009; Kajander 2007]. Here we focus on the development of one such TPR-peptide interaction for a practical application – affinity purification. We illustrate the general utility of our designed protein interaction. Furthermore, this example highlights how basic research on protein-peptide interactions can lead to the development of novel reagents with important practical applications.

Keywords

Protein design; repeat protein; protein-protein interactions; antibody; affinity reagent; protein purification

Introduction

The design and engineering of repeat proteins has been particularly successful [1–10]. The natural function of repeat proteins is to bind to macromolecules, including other proteins. Their high surface area to volume ratio, compared to globular proteins, presumably facilitates such interactions [11]. Manipulating the binding properties of natural repeat proteins is particularly interesting because it can lead to the creation of novel proteins with unique binding properties [12]. Here, we describe a designed repeat protein interaction whose binding characteristics vary substantially from its parent protein and make it

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particularly appealing as an affinity capture reagent. We use this interaction as an example of how to move a new repeat protein to a practical application.

Biochemical and cell biological research frequently require the purification of a desired protein from a complex cell lysate. The most powerful purification strategies use affinity reagents to isolate target proteins in a single step. Typically, these reagents consist of a peptide epitope tag that is fused to a target protein (the “prey”) and an antibody that binds to the epitope (the “bait”). The high affinity and specificity of antibodies make them powerful affinity reagents. However, these favorable properties are counterbalanced by the harsh elution conditions that are required to disrupt the interactions. These stringent conditions, such as extremes of pH, may denature either the target protein of interest or the antibody. Indeed, antibodies are often not reusable because of this irreversible denaturation. Furthermore, they are often extremely expensive, especially if produced using hybridoma technologies. See Table 1 for a more detailed comparison of different affinity purification strategies.

Tetratricopeptide repeat affinity proteins or TRAPs recognize short extended peptides [11]. We previously designed a TRAP, which we call affiTRAP, whose peptide binding properties make it ideally suited for protein purification [5]. Namely, it specifically binds to a short peptide, which we refer to as ‘Tag’. Furthermore, the affiTRAP-Tag interaction resists washing yet it can be disrupted using relatively mild conditions. Unlike antibodies, the affiTRAP is small and easy to produce in high yield from *E. coli*. These properties make it a more convenient and less expensive alternative to antibody-based affinity purification. Our system consists of a short five-residue peptide (sequence MEEVF), which is fused to the C-terminus of a target protein and the affiTRAP, which we immobilize onto resin. Here we describe the development of the affiTRAP-Tag system for protein purification in bacteria and mammalian cells. This system will be a useful addition to the biotechnology repertoire of many researchers.

Origins of the affiTRAP-Tag system

During the characterization of a number of TRAP-peptide complexes, we observed one such interaction that was extremely resistant to washing. These properties make it compatible with use in protein purification. Therefore, we sought to further optimize conditions for its use in protein purification.

Identification of wash and elution conditions

First, we sought to identify suitable wash and elution conditions using a simple GST pull-down assay with purified components (refer to Fig 1A). The affiTRAP-Tag interaction is extremely resistant to high salt (NaCl) concentrations (Fig 1B) [5]. We also observed that the affiTRAP-Tag interaction is resistant to pH extremes (pH 3 to pH 9) (data not shown). Table 2 summarizes a number of recommended wash conditions based on our extensive characterization of this interaction in a purification setting.

The main challenge was to identify suitable elution conditions. Specifically, we sought mild elution conditions that disrupt the affiTRAP-Tag interaction. We systematically tested

different combinations of anions and cations in the Hofmeister series [13]. We identified two conditions that effectively disrupt the interaction – magnesium chloride (MgCl_2) and potassium thiocyanate (KSCN) (Fig 1C). We also explicitly tested whether MgCl_2 denatures the affiTRAP using circular dichroism. It does not (data not shown). The absorbance of KSCN precluded similar experiments; however, we observed that many fluorescent proteins retained their fluorescence after elution with this salt. Table 3 summarizes a number of recommended elution conditions.

Applying the optimized conditions to a mock purification

Having established appropriate wash and elution conditions, we next sought to investigate the use of the affiTRAP-Tag system in controlled conditions that mimic a purification setting. In Fig 2A, we verify that hexahistidine tagged affiTRAP immobilized on Ni-NTA resin is able to retrieve a GST-Tag fusion protein from an *E. coli* cell lysate. We also confirm that the affiTRAP-Tag interaction is specific. The affiTRAP resin does not recover GST alone or GST-Tag* (Tag* contains a point mutation that does not bind to affiTRAP *in vitro*). These controls confirm that the affiTRAP-Tag system functions as anticipated. Namely, that both the affiTRAP and its cognate Tag are required for purification.

To investigate the generality of the method, we performed a similar set of experiments using a different immobilization strategy. We immobilized the affiTRAP on glutathione resin by using a GST-affiTRAP fusion protein (Fig 2B). We confirm that affiTRAP immobilized in this fashion is able to isolate tagged protein (mCherry-Tag) from an *E. coli* cell lysate. Again, we showed that both the affiTRAP and its cognate Tag are required for purification. To summarize, our system is not dependent on a particular immobilization strategy, adding to its potential versatility.

For our system to be broadly useful for protein purification, it must be able to isolate target proteins in high yield and purity. We tested the ability of an affiTRAP column (made by immobilizing hexahistidine tagged affiTRAP to Ni-NTA resin as described above) to recover tagged protein from an *E. coli* cell lysate (Fig 3A). The affiTRAP resin effectively captures the tagged protein and it is released efficiently through the addition of elution buffer (0.5 M MgCl_2) (Fig 3B). Furthermore, the tagged protein is recovered in relatively high purity.

Purification from mammalian cells

Finally, perhaps the most common use of affinity reagents is in the context of mammalian cell lysates. We therefore investigated the efficacy of the affiTRAP-Tag system in purification from mammalian cells. HEK293T cells were transiently transfected with either GFP or GFP-Tag. We used affiTRAP bound to either glutathione or Ni-NTA resin. AffiTRAP immobilized on either resin is able to retrieve tagged GFP from mammalian cell lysate (Fig 4A and B). Again, the affiTRAP resins do not recover untagged GFP indicating that the interaction is dependent on the affiTRAP-Tag interaction (Fig 4). These results illustrate the broad applicability of the affiTRAP-Tag system.

Conclusions

Protein design has reached an exciting stage because new proteins are now being used in myriad practical applications, including diagnostics, therapeutics, and nanotechnology [14–17].

Here we develop an interesting designed protein interaction into a potentially widely applicable research tool. For any affinity reagent to be practically useful, it must bind to its target specifically and robustly. In other words, it must be resistant to washing so that other proteins in a cell lysate can be readily removed. Conversely, one must be able to disrupt this interaction and release the tagged protein of interest. Ideally, the elution conditions are gentle enough so that the affinity reagent is not denatured and can be reused multiple times. Finally, the cost of the affinity reagent should be as low as reasonably possible. Here, we have shown that the properties of the affiTRAP-Tag interaction meet all of these criteria. We anticipate that this tool will be of great use to other researchers in many situations. We also anticipate that new users will build on this system by adding additional features and embellishments.

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Abbreviations

TPR	tetratricopeptide repeat
TRAP	tetratricopeptide repeat affinity protein

References

1. Cortajarena AL, Mochrie SG, Regan L. Modulating repeat protein stability: the effect of individual helix stability on the collective behavior of the ensemble. *Prot Sci*. 2011; 20:1042–1047.
2. Main ERG, Xiong Y, Cocco MJ, D'Andrea L, Regan L. Design of Stable α -Helical Arrays from an Idealized TPR Motif. *Structure*. 2003; 11:497–508. [PubMed: 12737816]
3. Cortajarena ALL, T Y, Hochstrasser M, Regan L. Designed proteins to modulate cellular networks. *ACS Chem Biol*. 2009; 5:545–552.
4. Cortajarena ALY,F, Regan L. Designed TPR modules as novel anticancer agents. *ACS Chem Biol*. 2008; 3:161–166. [PubMed: 18355005]
5. Jackrel ME, Valverde R, Regan L. Redesign of a protein-peptide interaction: characterization and applications. *Prot Sci*. 2009; 18:762–774.
6. Kajander T, Cortajarena AL, Mochrie S, Regan L. Structure and stability of designed TPR protein superhelices: unusual crystal packing and implications for natural TPR proteins. *Acta Crystallog D*. 2007; 63:800–811.

7. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grutter MG, Pluckthun A. High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol.* 2004; 22:575–582. [PubMed: 15097997]
8. Park K, Shen BW, Parmeggiani F, Huang PS, Stoddard BL, Baker D. Control of repeat-protein curvature by computational protein design. *Nat Struct Biol.* 2015; 22:167–174.
9. Parker R, Mercedes-Camacho A, Grove TZ. Consensus design of a NOD receptor leucine rich repeat domain with binding affinity for a muramyl dipeptide, a bacterial cell wall fragment. *Prot Sci.* 2014; 23:790–800.
10. Regan L, Caballero D, Hinrichsen MR, Virrueta A, Williams DM, O'Hern CS. *Protein Design: Past, Present and Future.* Biopolymers. 2015
11. Sawyer N, Speltz EB, Regan L. NextGen protein design. *Biochem. Soc Trans.* 2013; 41:1131–1136.
12. Grove TZO, C O, Forster JD, Dufresne ER, Regan L. Stimuli-responsive smart gels realized via modular protein design. *J Am Chem Soc.* 2010; 132:14024–14026. [PubMed: 20860358]
13. Hofmeister F. Zur Lehre von der Wirkung der Salze. *Zweite Mittheilung.* 1888; 24:247–260.
14. Strauch EMF, S J, Baker D. Computational design of a pH-sensitive IgG binding protein. *Proc Natl Acad Sci.* 2014; 111:675–680. [PubMed: 24381156]
15. Zahnd C, Kawe M, Stumpp MT, de Pasquale C, Tamaskovic R, Nagy-Davidescu G, Dreier B, Schibli R, Binz HK, Waibel R, Pluckthun A. Efficient tumor targeting with high-affinity designed ankyrin repeat proteins: effects of affinity and molecular size. *Cancer Res.* 2010; 70:1595–1605. [PubMed: 20124480]
16. Gradisar H, Bozic S, Doles T, Vengust D, Hafner-Bratkovic I, Mertelj A, Webb B, Sali A, Klavžar S, Jerala R. Design of a single-chain polypeptide tetrahedron assembled from coiled-coil segments. *Nat Chem Biol.* 2013; 9:362–366. [PubMed: 23624438]
17. Pluckthun A. Designed ankyrin repeat proteins (DARPs): binding proteins for research, diagnostics, and therapy. *Annu Rev Pharmacol.* 2015; 55:489–511.
18. Raines RTM, M, Oosbree TRV, Mierendorf RC. The S-tag fusion system for protein purification. *Method Enzymol.* 2000; 326:362–376.
19. Fuchs SM, Raines RT. Polyarginine as a multifunctional fusion tag. *Prot Sci.* 2005; 14:1538–1544.
20. Kimple MEAS, J. Affinity tag for protein purification and detection based on the disulfide-linked complex of InaD and NorpA. *BioTechniques.* 2002; 33:578–590. [PubMed: 12238768]
21. Speltz EB, Regan L. White and green screening with circular polymerase extension cloning for easy and reliable cloning. *Prot Sci.* 2013; 22:859–864.
22. Studier FW. Protein production by auto-induction in high-density shaking cultures. *Protein Express Purif.* 2005; 41:207–234.
23. Zhao C, Brown RS, Chase AR, Eisele MR, Schlieker C. Regulation of Torsin ATPases by LAP1 and LULL1. *Proc Natl Acad Sci USA.* 2013; 110:E1545–1554. [PubMed: 23569223]

Figure 1. Determination of suitable elution and wash conditions for the affiTRAP-Tag interaction

A. Schematic representation of the pulldown assay using purified components.

The affinity resin consists of GST-Tag fusion protein (green circle) immobilized on glutathione resin (grey circle). The affinity resin is incubated with the affiTRAP (blue circle). Unbound affiTRAP is removed by washing. The resin is incubated with a test elution buffer. The remainder of the GST-Tag and bound affiTRAP are released from the resin by the addition of SDS PAGE sample buffer and boiling. Both the test elution buffer ('elution') and SDS-PAGE sample buffer ('SDS') are analyzed by SDS-PAGE. Below we show a cartoon representation for an unsuitable (left) versus suitable (right) elution buffer.

B. NaCl is an excellent wash agent. Testing the resilience of the affiTRAP-Tag interaction to increasing concentrations of NaCl. The concentration of NaCl (M) (in 50 mM Tris, pH 7.4) is indicated above each lane.

C. Identification of anions and cations in the Hoffmeister series that are good elution agents. The identity of the salt (each at 0.5 M) added to PBS buffer (10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl, pH 7.4) is indicated above each lane. The two suitable elution conditions are highlighted in orange text.

Figure 2. The specificity of the affiTRAP-Tag system

Left: Schematic illustration of the baits and prey used. In both panels the baits are: resin alone (grey circles); affiTRAP (blue ribbon representation of the protein structure) immobilized on the resin; and GST (blue pentagon) immobilized on the resin. The prey proteins are represented by green pentagons, with tags as indicated.

A. AffiTRAP is immobilized on Ni-NTA resin via a hexahistidine tag. We used both Ni-NTA resin alone and affiTRAP immobilized on Ni-NTA resin as baits. We used three different prey proteins: GST (green pentagons), GST fused to a mutated Tag (Tag*); and GST fused to the cognate Tag. We tested the ability of each resin to pulldown each prey from cell lysate. On the right, we show SDS-PAGE analysis for each pulldown assay. The resin (bait) used for each pulldown is indicated above the lane; while the identity of the prey protein is indicated in a white box at the bottom of the gel. The release buffer comprises PBS with 0.25 M imidazole.

B. AffiTRAP is immobilized on glutathione resin via a GST tag. Three types of resin were used as bait: glutathione resin alone; GST immobilized on glutathione resin; the GST-affiTRAP fusion protein immobilized on glutathione resin. For prey, we used either mCherry fused to a mutated Tag (Tag*) or mCherry fused to the Tag. The release buffer comprises PBS with 0.05 M glutathione. The samples here are not boiled prior to loading so that mCherry fluorescence is retained in the gel. We show both the Coomassie stained gel and its corresponding fluorescence image. For both gels, prey protein is indicated by open arrows while bait protein is indicated by closed arrows.

Figure 3. Immobilized affiTRAP can be used to purify tagged proteins from bacterial cell lysate

A. Schematic representation of all the components of the protein purification system:

Hexahistidine tagged affiTRAP (blue ribbon representation) is immobilized on Ni-NTA resin (grey circles). Cell lysate (various purple shapes) containing a tagged prey protein (green pentagons) is added to the affinity resin. Prey protein is eluted with MgCl_2 buffer. AffiTRAP is released using imidazole buffer.

B. AffiTRAP purifies tagged protein efficiently. SDS-PAGE analysis of the GST-Tag purification from *E. coli* cell lysate. The contents of each lane are as indicated. The wash buffer comprises 50 mM Tris, pH 7.4, 0.3 M NaCl. The prey elution buffer comprises 100 mM MES, pH 6.5, 0.5 M MgCl_2 . The imidazole release buffer comprises 50 mM Tris, pH 7.4, 300 mM NaCl, 0.25 M imidazole. The location of prey protein in the gel is indicated by open arrows while bait protein is indicated by closed arrows.

Figure 4. Immobilized affiTRAP can be used to purify tagged proteins from mammalian cell lysate

In both gels, we compare the behavior of affiTRAP resin with lysate from mammalian cells expressing GFP (first four lanes) or GFP-Tag (last four lanes). The affiTRAP is immobilized on Ni-NTA (A) or glutathione (B) resin via a hexahistidine tag or GST tag, respectively. The prey elution buffer comprises PBS buffer with 0.5 M MgCl₂. The TRAP release buffer comprises PBS buffer with either 0.25 M Imidazole (A) or 0.05 M glutathione (B). The samples are not boiled prior to loading so that GFP fluorescence is retained in the gel. We show both the same gel stained with Coomassie Blue and its corresponding fluorescence image. Prey protein is indicated by open arrows while bait protein is indicated by closed arrows.

Table 1

Comparison of common protein purification strategies

Tag	Length (Da) ^α	Position	Binding Partner	Elution conditions	Cost ^δ (USD/mL)	Capacity (nmol/mL)	Reusable
MBP	42000	N or C	Amylose	Maltose	10.7 (NEB)	100–140	yes
GST	26000	N or C	Glutathione	Glutathione	26.3 (GE)	560	yes
S [18]	1749	N or C	S-protein	Protease, pH<2, guanidium, 3 M MgCl ₂	53 (Merck)	NA	no
Myc ^γ	1202	N or C	Antibody	High pH	212.5 (Pierce)	102–144	semi
HA ^γ	1102	N or C	Antibody	Free HA peptide	456 (Pierce)	60–150	semi
FLAG ^γ	1012	N or C	Antibody	EDTA, Glycine, or free peptide	438.5 (Sigma)	30 ^β	semi
His	840	N or C	Nickel NTA	0.1–25 M Imidazole	11.4 (Qiagen)	250–500 ^β	yes
Arg [19]	800	C	Cation Exchange	salt	NA	NA	yes
Strep	1000	N or C	streptactin	desthiobiotin	83 (Qiagen)	150 ^β	yes
CBD	5600	N or C	Chitin	SDS or guanidium HCL	3.8 (NEB)	48	semi
NorpA [20]	600	C	PDZ1	BME, DTT, glutathione		NA	semi
AffTag	654	C	AffTRAP	0.5 M KSCN, MgCl ₂	NA	NA	semi

^αMinimum length, linker or recommended linker not included

^βCalculated assuming a 20 kDa protein

^γData for antibody covalently cross-linked to agarose resin.

^δCost information was retrieved from company websites (see below) on April 22, 2015

NA indicates that this information is not available

NEB is New England Biolabs (Ipswich, MA); Qiagen (Valencia, CA); Merck (Kenilworth, NJ); Pierce (Waltham, MA); Sigma (St. Louis, MO); GE Healthcare Life Sciences (Piscataway, NJ)

Table 2

Recommended Wash Buffers

Buffer ^a	Additive
100 mM MES, pH 6.5 PBS ^β Water	–
	up to 1 M NaCl
	10% Glycerol
	10% MPD
	0.5 M xylitol
	0.5 M xylitol, 10% glycerol
	1 M KCl
	0.5 M MgSO ₄
	0.5 M (NH ₄) ₂ SO ₄
	0.5 M Mg(CH ₃ COO) ₂
	0.5 M KH ₂ PO ₄

^a Buffers can be used in combination with any of the additives listed

^β PBS is 10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl, pH 7.4

Table 3

Recommended Elution Buffers

Buffer	Additive ^a
100 mM MES, pH 6.5 50 mM Tris, pH 7.4 PBS ^β	0.5 M KSCN
100 mM MES, pH 6.5 50 mM Tris, pH 7.4 PBS ^β	0.5 M MgCl ₂
100 mM Citrate Buffer, pH <2.9	–
100 mM Glycine Buffer, pH <2.5	–

^a Additives can be used in combination with any of the buffers listed

^β PBS is 10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl, pH 7.4