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A peptide tag system for facile purification and single-molecule immobilization

Jin Huang, Stanislav S. Nagy, Akiko Koide, Ronald S. Rock^{*}, and Shohei Koide^{*}

Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637, USA.

Abstract

A peptide fusion tag and accompanying recombinant capture reagents have been developed based on the peptide-PDZ domain interaction and affinity clamps, a new class of affinity reagent. This system allows for single-step purification under mild conditions and stable capture of a tagged protein. The sub-nanomolar affinity, high force resistance (>30 pN), small size (~25kDa, ~1/6 of IgG) and monomeric nature of the affinity clamp are all superior features for many applications, in particular single-molecule measurements.

Genetic fusion tags and accompanying capture reagents facilitate detection, purification and immobilization of recombinant proteins, and thus they have become an integral component in biochemical, biophysical and cellular investigations (1). Short peptide tags, often called epitope tags, are minimally invasive and useful for detection and purification. Folded protein tags often serve additional roles in increasing the solubility and expression yields. Together, available tag systems satisfy the requirements for standard applications in protein expression, detection and purification.

Single-molecule measurements are uniquely powerful in their ability to reveal the molecular mechanisms underlying motility, conformational changes and force generation. Fusion tags are routinely used for immobilization and detection in single-molecule measurements but this type of research poses demanding requirements for fusion tags and their accompanying capture reagents. (i) A fusion tag should be short so as to minimally affect the structure and oligomerization state of the protein of interest. (ii) The interaction between a tag and its capture reagent must be of high affinity so that it is stably formed even at very low protein concentrations (typically ~10 pM). (iii) A capture reagent should be monomeric and highly specific. (iv) Multiple, mutually orthogonal tag/capture reagent systems should be available. These requirements render a majority of commonly used tag/capture reagent systems unsuitable for single-molecule measurements.

The current standard is to combine the biotinylation tag/(strept)avidin system with a monoclonal antibody (mAb) system (2–5). Although the biotinylation tag/(strept)avidin system is widely used due to its high affinity and specificity(6), (strept)avidin is a tetrameric protein that can potentially bring multiple molecules to close proximity (Fig. 1a). A GFP/anti-GFP mAb system offers some unique advantages, but the complex is large and can also force artificial dimerization, making it a suboptimal solution (Fig. 1a). Furthermore, we have experienced batch-to-batch inconsistency of monoclonal antibodies. Recent commercial

^{*}To whom correspondence should be addressed. Phone: (773)702-5073 (SK) or (773)702-0716. Fax: (773)702-0439 (SK). skoide@uchicago.edu (SK) or rock@uchicago.edu (RSR). A request for materials should be addressed to SK.

Supporting Information Available: Experimental Methods, Supplementary Figure and Supplementary Movies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

preparations of anti-GFP antibodies contained actin-binding contaminants that interfered with our work on myosin motors. We therefore sought an alternative that would combine high affinity with high specificity, and would also complement the streptavidin-biotin links that we use elsewhere in our experimental systems.

Here, we developed a short peptide tag/capture reagent system that addresses all of the requirements outlined above. It is based on a new type of recombinant affinity reagents, termed "affinity clamps", that we have recently developed (7). Affinity clamps are small (~25 kDa) recombinant proteins that are engineered through structure-guided directed evolution. One of such affinity clamps, called ePDZ-b1, is a fusion protein consisting of a circularly permuted PDZ domain of human erbin and a phage display-optimized fibronectin type III domain (FN3). It binds to an eight-residue peptide segment located at the C-terminal extreme of the human ARVCF protein with single-nM dissociation constant (K_d) (7). This interaction was highly specific, as demonstrated by the ability of the affinity clamp to specifically detect the ARVCF protein in Western-blotting of cell lysate (7). Subsequently, we have characterized the peptide sequence preference of ePDZ-b1 from which we designed a nonnatural sequence with even higher affinity (sub-nM K_d) (8).

The fusion tag designed in the present work, termed "C-tag", further encodes a thrombin cleavage site that overlaps the affinity clamp-binding sequence (Fig. 1b). The C-tag sequence attached C-terminal to yeast SUMO protein bound ePDZ-b1 with a sub-nM K_d and a dissociation half life of a few hours as measured using surface plasmon resonance at 25 °C (Supplementary Fig. 1a). This level of affinity is 3–4 orders of magnitude greater than those for FLAG/antibody and c-myc/antibody (both ~400 nM K_d) (9,10) and His₆-tag/immobilized metal (~10 μ M) (11). The tag can be readily cleaved with thrombin as expected (Supplementary Fig. 1c).

We then critically tested the C-tag system as a general protein handle for single-molecule microscopy. We constructed a motor protein, myosin X(12,13), tagged with the FLAG tag and the C-tag at the C-terminus (Fig. 1c). In addition, we constructed an ePDZ-b1 variant with a single Cys residue to which a fluorescent dye, Cy5, was chemically conjugated (see Methods in Supplementary Information).

We achieved specific and stable immobilization of myosin X to a solid support via the C-tag/affinity clamp. In gliding filament assays, we observed smooth and continuous actin filament gliding on coverslips precoated with a high concentration of the ePDZ-b1 protein to which C-tagged myosin X was added (Fig. 2a and Supplementary Movie 1). The actin filaments moved at 0.33 ± 0.02 nm/s ($n = 50$), similar to what we previously observed with myosin X fused to GFP and anti-GFP for surface attachment ($0.25 - 0.35$ μ m/s) (13), suggesting that the C-tagged myosin X was fully active. As we reduced the concentration of the affinity clamp used for coating, fewer actin filaments moved (with increasing filament flexibility), until the surface density of motor was too low to support the movement of actin filaments. This motility behavior upon dilution suggests that myosin X is attached to the surface only through the C-tag/affinity clamp linkage with no cross-reactivity of the affinity clamp to the actin filaments.

In a TIRF motility assay using the Cy5-labeled Affinity Clamp with the C-tagged myosin X motor, we observed single fluorescent particles, corresponding to myosin X motors carrying the labeled affinity clamp, move along actin tracks (Fig. 2b and Supplementary Movie 2). The run lengths were similar to our previous measurements utilizing calmodulin-exchanged myosin X (13). We found no detectable staining of the actin fibers, indicating high specificity of the affinity clamp.

In optical trapping assays, we found the C-tag/affinity clamp to be a robust handle that can withstand significant forces. In a three-bead optical trapping assay, we saw the same motor

repeatedly bound along single actin filaments (Fig. 2c), indicating that the motor remained attached to the surface for > 1h (the total duration for assay setup and recording). To test force resistance, after the motor bound to the filament we applied a large force (~30 pN) to the motor-affinity clamp complex by moving the stage (Fig. 2d). At these superstall forces, the myosin X stepped backwards resulting in reduced effective forces and also occasionally detached from the actin filament resulting in the elimination of the offset and the applied forces. We were able to repeatedly pull on the same molecule, showing that the immobilized myosin X molecule did not dissociate from the platform bead over the course of the experiment. Together these data indicate that the linkage through the C-tag system was maintained over an extended period even when a large force was applied.

The C-tag can also be used for affinity purification. Here, we exploited the fact that the C-tag also binds to the wild-type erbin PDZ domain with low μM K_d , a level of affinity appropriate for elution from immuno-affinity purification (14). We prepared affinity capture resin by immobilizing erbin PDZ on agarose beads. We used a high-affinity peptide to erbin PDZ (14) with a distinct sequence from the C-tag (termed "elution peptide" hereafter) to competitively release a captured protein. This system effectively purified the C-tagged SUMO protein expressed in *E. coli* in a single step (Supplementary Fig. 1b). This resin had a high binding capacity (~10 mg C-tagged SUMO purified with 1 ml resin). A convenient feature of this system is that the elution peptide does not bind tightly to ePDZ-b1 (data not shown), eliminating the necessity to remove it prior to immobilizing a purified C-tagged protein to the affinity clamp. The purity of myosin X tagged with both FLAG and C-tag was comparable to that of the same protein purified with the anti-FLAG antibody affinity column, although a major impurity from anti-FLAG purification was absent. (Fig. 1d). We noted a lower level of recovery of myosin X from the C-tag purification. This is probably because we have already optimized the anti-FLAG purification and the bivalent interaction of dimeric myosin X with the capture resin makes it harder to elute the captured protein.

The PDZ resin can be regenerated repeatedly by washing with urea or guanidine hydrochloride (Figure S1). As expected from the absence of disulfide bonds in the PDZ domain, exposure to dithiothreitol even with guanidine hydrochloride did not cause a detectable change in the performance of the resin. An advantage of our affinity resin is that the PDZ domain is produced in high yield in *E. coli* (~50 mg/liter culture), which is more economical than monoclonal antibody production from hybridoma cells.

In conclusion, the C-tag system offers many advantages over existing fusion tag systems. As a fully recombinant system, affinity clamps can be easily reformatted for the specific needs of each application. We note that the C-tag must be attached to the C-terminus of a target protein. While this requirement presents some limitations, it is also the origin of the exquisite specificity of the C-tag/affinity clamp linkage and it can be used to selectively purify the full-length protein. We speculate that the affinity clamp can be stably expressed in cells as a GFP fusion protein, which may be useful in live cell imaging. Together, we believe that the C-tag system will find broad utility in biophysical, biochemical and cell biology applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Waugh DS. Making the most of affinity tags. *Trends Biotechnol* 2005;23:316–320. [PubMed: 15922084]
2. Kellermayer MS, Smith SB, Granzier HL, Bustamante C. Folding-unfolding transitions in single titin molecules characterized with laser tweezers. *Science* 1997;276:1112–1116. [PubMed: 9148805]
3. Tskhovrebova L, Trinick J, Sleep JA, Simmons RM. Elasticity and unfolding of single molecules of the giant muscle protein titin. *Nature* 1997;387:308–312. [PubMed: 9153398]
4. Revyakin A, Ebright RH, Strick TR. Single-molecule DNA nanomanipulation: improved resolution through use of shorter DNA fragments. *Nat Methods* 2005;2:127–138. [PubMed: 16156080]
5. Rock RS, Rice SE, Wells AL, Purcell TJ, Spudich JA, Sweeney HL. Myosin VI is a processive motor with a large step size. *Proc Natl Acad Sci U S A* 2001;98:13655–13659. [PubMed: 11707568]
6. Stayton PS, Freitag S, Klumb LA, Chilkoti A, Chu V, Penzotti JE, To R, Hyre D, Le Trong I, Lybrand TP, Stenkamp RE. Streptavidin-biotin binding energetics. *Biomol Eng* 1999;16:39–44. [PubMed: 10796983]
7. Huang J, Koide A, Makabe K, Koide S. Design of protein function leaps by directed domain interface evolution. *Proc Natl Acad Sci U S A* 2008;105:6578–6583. [PubMed: 18445649]
8. Huang J, Makabe K, Biancalana M, Koide A, Koide S. Structural basis for exquisite specificity of affinity clamps, synthetic binding proteins generated through directed domain-interface evolution. *J Mol Biol* 2009;392:1221–1231. [PubMed: 19646997]
9. Einhauser A, Jungbauer A. Affinity of the monoclonal antibody M1 directed against the FLAG peptide. *J Chromatography A* 2001;921:25–30.
10. Krauss N, Wessner H, Welfle K, Welfle H, Scholz C, Seifert M, Zubow K, Ay J, Hahn M, Scheerer P, Skerra A, Hohne W. The structure of the anti-c-myc antibody 9E10 Fab fragment/epitope peptide complex reveals a novel binding mode dominated by the heavy chain hypervariable loops. *Proteins* 2008;73:552–565. [PubMed: 18473392]
11. Dorn IT, Neumaier KR, Tampe R. Molecular recognition of histidine-tagged molecules by metal-chelating lipids monitored by fluorescence energy transfer and correlation spectroscopy. *J Am Chem Soc* 1998;120:2753–2763.
12. Berg JS, Derfler BH, Pennisi CM, Corey DP, Cheney RE. Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin. *J Cell Sci* 2000;113(Pt 19):3439–3451. [PubMed: 10984435]
13. Nagy S, Ricca BL, Norstrom MF, Courson DS, Brawley CM, Smithback PA, Rock RS. A myosin motor that selects bundled actin for motility. *Proc Natl Acad Sci U S A* 2008;105:9616–9620. [PubMed: 18599451]
14. Skelton NJ, Koehler MF, Zobel K, Wong WL, Yeh S, Pisabarro MT, Yin JP, Lasky LA, Sidhu SS. Origins of PDZ domain ligand specificity. Structure determination and mutagenesis of the Erbin PDZ domain. *J Biol Chem* 2003;278:7645–7654. [PubMed: 12446668]

Figure 1.

Design of the C-tag system and its use in affinity purification. **a)** A comparison of the C-tag and its capture reagents (affinity clamp and PDZ) with commonly used tag/capture reagents in single-molecule measurements, biotin/streptavidin and GFP/antibody. The molecules are drawn to the scale. C-tag and biotin are shown in yellow. Note that the Fab represents ~1/3 of the full antibody molecule that is shown as a scheme. Because the structure of a GFP - antibody complex is not known, an unrelated Fab structure is shown. Protein Data Bank entries 3CH8, 1STP, 1S6Z and 1DQJ were used. **b)** The amino acid sequence of the C-tag. The recognition sequence for the affinity clamp is shown in bold and the thrombin recognition sequence is underlined. **c)** Schematic drawing of the myosin X construct used in this work. Red portions

corresponding to the myosin X heavy chain dimer is shown in red, and calmodulin as the orange circles. The tags are attached to the C-terminus of myosin X. **d**) Affinity purification of myosin X tagged with both FLAG and C-tag. SDS-PAGE stained with Coomassie Brilliant Blue showing lysate of Sf9 cells expressing myosin X ("lysate"), sample purified with the PDZ affinity resin ("C-tag") and the anti-FLAG antibody resin ("FLAG"), and molecular weight markers (the rightmost lane).

Figure 2. Applications of the C-tag system to single molecule measurements

a) The C-tag system immobilizes motors for gliding filament motility assays. The ePDZ-b1 protein (shown as “U”) was adsorbed on a nitrocellulose coated glass coverslip surface, then C-tagged myosin X was added. The myosin X bound to the surface through its tail, exposing the motor domains. Actin filaments labeled with rhodamine-phalloidin (white) were then introduced and bound to motor in the absence of ATP, after which 2 mM ATP was added to initiate motility. Trajectories of actin filaments moving along the surface illustrated by overlapping 6 frames from a movie in different colors. Scale bar, 10 μm . See also Supplementary Movie 1. **b)** The C-tag system labels motors for TIRF motility assays. A Cy5-labeled ePDZ-b1 protein (green) was mixed with ~50 pM C-tagged myosin X and 2 mM ATP,

and then introduced to a microscopy flow cell with fascin-actin bundles adhered to the coverslip surface via a biotin/neutravidin linkage. Single processive motor runs were imaged by TIRF microscopy. One such event is represented here with a series of frames separated by 2 seconds. Scale bar, 1 μm . See also Supplementary Movie 2. **c)** The C-tag system immobilizes motors for single-molecule optical trapping assays. Single C-tagged myosin X molecules were attached to a platform bead that had been coated with the ePDZ-b1 protein. An optically trapped actin dumbbell was brought into the proximity of the myosin X. The trace shows the position of one of the optically trapped beads along the axis of the actin filament. As the motor interacted with the actin filament, deflections in the bead position trace were observed (orange). **d)** The C-tag system withstands a high external force. In optical trapping assays as in (D), the holding force of the affinity clamp was tested by rapidly moving the stage by approximately 300 nm as the motor interacted with the actin filament, displacing the dumbbell out of the traps and imposing a large force (~ 30 pN) on the actin-motor-clamp complex. The motor then detached from actin and the dumbbell relaxed to its original position. The repeatedly observed events (five shown) indicate that the clamp-motor linkage remained intact over the course of the measurement.