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Rapid Report

A Peptide Tag System for Facile Purification and Single-Molecule Immobilization†

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ABSTRACT: A peptide fusion tag and accompanying recombinant capture reagents have been developed on the basis of 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 subnanomolar affinity, high force resistance (> 30 pN), small size (~25 kDa, approximately one-sixth of the size 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 because of its high affinity and specificity (*6*), (strept)avidin is a tetrameric protein that can potentially bring multiple molecules into the proximity of one another (Figure 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 (Figure 1a). Furthermore, we have experienced batch-to-batch inconsistency of monoclonal antibodies. Recent commercial 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 reagent, termed an "affinity clamp", that we have recently developed (*7*). Affinity clamps are small (~25 kDa) recombinant proteins that are engineered through structure-guided directed evolution. One such affinity clamp, called ePDZ-b1, is a fusion protein consisting of a circularly permutated 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 a single-nanomolar 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 the cell lysate (*7*). Subsequently, we have characterized the peptide sequence preference of ePDZ-b1 from which we designed a nonnatural sequence with an even higher affinity (subnanomolar K_d) (*8*).

The fusion tag designed in this work, termed "C-tag", further encodes a thrombin cleavage site that overlaps the affinity clamp-binding sequence (Figure 1b). The C-tag sequence attached C-terminally to the yeast SUMO protein bound to ePDZ-b1 with a subnanomolar K_d and a dissociation half-life of a few hours as measured using surface plasmon resonance at 25 °C (Figure 1a of the Supporting Information). This level of affinity is 3−4 orders of magnitude greater than those for FLAG−antibody and c-myc−antibody systems (both with K_d values of ~400 nM) (*9*, *10*) and the His_6 tag−immobilized metal system ($K_d \sim 10\ \mu M$) (*11*). The tag can be readily cleaved with thrombin as expected (Figure 1c of the Supporting Information).

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 (Figure 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 the Supporting 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

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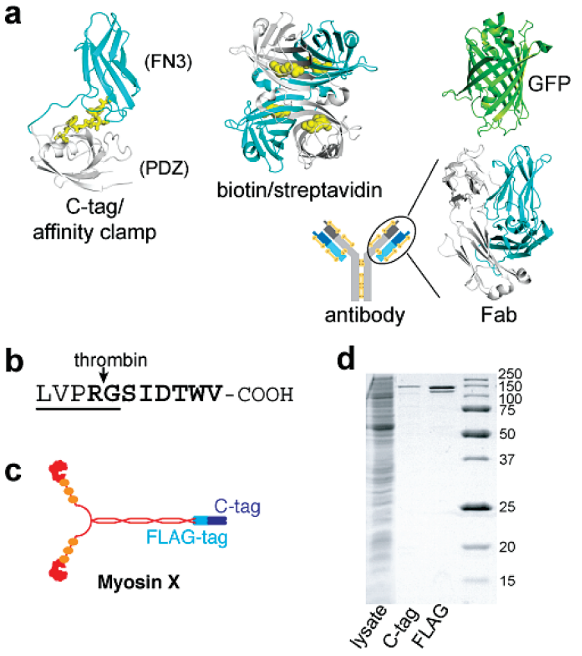

FIGURE 1: Design of the C-tag system and its use in affinity purification. (a) A comparison of the C-tag and its capture reagents (affinity clamp and PDZ) with commonly used tag−capture reagents in single-molecule measurements, biotin−streptavidin and GFP−antibody complexes. The molecules are drawn to scale. C-Tag and biotin are colored yellow. Note that the Fab represents approximately one-third 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) 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 are colored red, and calmodulin is depicted as 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 the lysate of Sf9 cells expressing myosin X (lysate), the sample purified with the PDZ affinity resin (C-tag) and the anti-FLAG antibody resin (FLAG), and molecular weight markers (the rightmost lane).

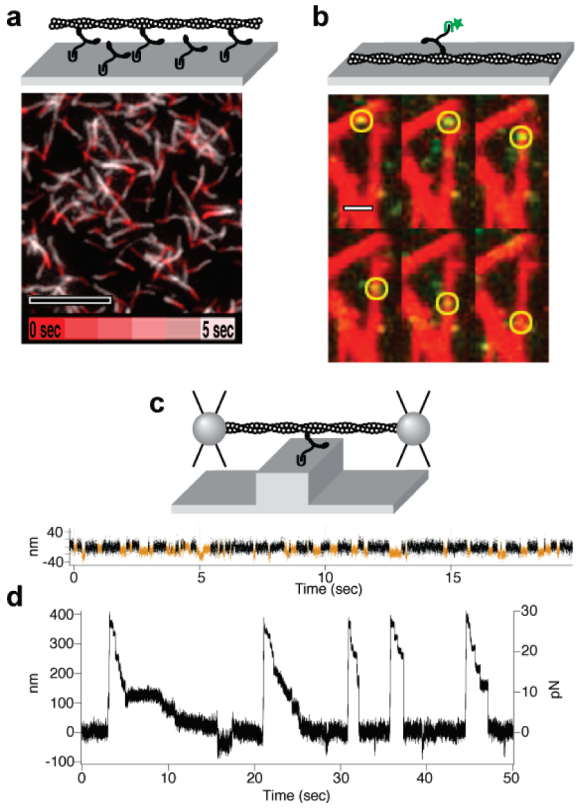

FIGURE 2: Applications of the C-tag system to single-molecule measurements. (a) The C-tag system immobilizes motors for gliding filament motility assays. The ePDZ-b1 protein (shown as U) was adsorbed on a nitrocellulose-coated glass coverslip surface, and then C-tagged myosin X was added. The myosin 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 the motor in the absence of ATP, after which 2 mM ATP was added to initiate motility. Trajectories of actin filaments moving along the surface are illustrated by overlapping six frames from a movie in different colors. The scale bar is 10 μm. See also Movie 1 of the Supporting Information. (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 the labeled protein was introduced into a microscopy flow cell with fascin−actin bundles that 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 s. The scale bar is 1 μm. See also Movie 2 of the Supporting Information. (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 strong external force. In optical trapping assays as in panel 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 from 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.

(Figure 2a and Movie 1 of the Supporting Information). The actin filaments moved at a rate of 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 μm/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 the 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 (Figure 2b and Movie 2 of the Supporting Information). 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 the 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 (Figure 2c), indicating that the motor remained attached to the surface for > 1 h (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 (Figure 2d). At these superstall forces, the myosin X stepped backward, 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 a low micromolar 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 peptide with a high affinity for 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 *Escherichia coli* in a single step (Figure 1b of the Supporting Information). This resin had a high binding capacity (~10 mg of C-tagged SUMO purified with 1 mL of 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 immobilization of 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 (Figure 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 1 of the Supporting Information). 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/L of culture), which is more economical than production of the monoclonal antibody 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.

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.

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