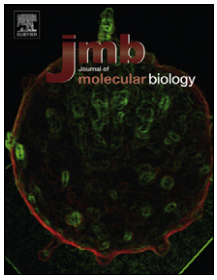

A New Versatile Immobilization Tag Based on the Ultra High Affinity and Reversibility of the Calmodulin–Calmodulin Binding Peptide Interaction

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Abstract

Reversible, high-affinity immobilization tags are critical tools for myriad biological applications. However, inherent issues are associated with a number of the current methods of immobilization. Particularly, a critical element in phage display sorting is functional immobilization of target proteins. To circumvent these problems, we have used a mutant (N5A) of calmodulin binding peptide (CBP) as an immobilization tag in phage display sorting. The immobilization relies on the ultra high affinity of calmodulin to N5A mutant CBP (RWKKNFIAV-SAANRFKKIS) in presence of calcium (K_D ~ 2 pM), which can be reversed by EDTA allowing controlled "capture and release" of the specific binders. To evaluate the capabilities of this system, we chose eight targets, some of which were difficult to overexpress and purify with other tags and some had failed in sorting experiments. In all cases, specific binders were generated using a Fab phage display library with CBP-fused constructs. K_D values of the Fabs were in subnanomolar to low nanomolar (nM) ranges and were successfully used to selectively recognize antigens in cell-based experiments. Some of these targets were problematic even without any tag; thus, the fact that all led to successful selection endpoints means that borderline cases can be worked on with a high probability of a positive outcome. Taken together with examples of successful case specific, high-level applications like generation of conformation-, epitope- and domain-specific Fabs, we feel that the CBP tag embodies all the attributes of covalent immobilization tags but does not suffer from some of their well-documented drawbacks.

Introduction

High-performance affinity tags play indispensable roles in almost all areas of biological research. Affinity tags are used in a variety of experiments including affinity purifications, Western blots, immunoprecipitations, flow cytometry, ELISAs, directed evolution experiments, biophysical measurements, immunofluorescence and many other biological applications. There is no tag that is universal; they all have their own strengths and weaknesses depending upon the application. Some of them such as His- [1,2], FLAG- [3], cMyc- [4] and V5- [5] tags have broader versatility, while others like Avi-tag [6], Fc-fusion tag [7] and ZZ-tag [8] are designed to perform a specific function with high fidelity. They cover a spectrum of characteristics from which a researcher can tune the tag for desired properties and ease of use. Some among these, for instance, the purification tags, are purposed to be reversible, while others are designed to form virtually covalent interactions with the target substrate. Thus, in most cases, the researcher is faced with trade-offs that need to be weighed before properly matching the tag with the application that it is being used for.

A case in point is tags that are used for antigen immobilization during directed evolution display experiments, particularly phage display. Phage display library sorting requires immobilization of the target antigen on a solid support in its native functional form [9] followed by vigorous washing steps to eliminate the undesired nonspecific binding effects. Purification tags like His- or FLAG-tags are too weak to make them viable candidates [1–3]. For

phage display, the most commonly used are tags that are based on the virtually irreversible biotin-streptavidin (SA) interaction [10–12]. This requires biotinylating the protein antigen either through chemical modification of the amine and sulfhydryl groups of amino acid residues or via an enzymatic biotinylation tag (Avi-tag), commonly introduced either at N-terminus or at C-terminus of the expression construct [12]. However, both these approaches have their shortcomings.

A frequently encountered issue with chemical biotinylation is that the process has to be very carefully optimized for each of the target as overbiotinylation drastically changes the surface properties of the target and often leads to partial denaturation and loss of activity of the targets. Moreover, the choice of the biotinylation reagent is especially crucial for certain classes of targets, especially for enzymes containing cysteine or lysine, which are the primary sites for chemical biotinylation. Harsh elution conditions with either high or low pH are generally used to recover phages bound to immobilized targets chemically biotinylated using noncleavable biotinylation reagents [13]. These nonspecific elution techniques risk the enrichment of background binders, thereby reducing the chance to obtain the desired specific clones. Some of the commercially available cleavable biotinylation reagents utilize the reduction of disulfide bond necessitating the storage and handling of the biotinylated proteins in an oxidizing environment. This is detrimental for proteins that are rich in surface-exposed and catalytic cysteine residues that require the presence of reducing agent in storage conditions and such nonessential reactive cysteine residues must be eliminated by mutation or chemical modification before chemical biotinylation [14].

An alternate to the chemical approach is enzymatic biotinylation where the protein is selectively biotinylated at a specific lysine residue in the sequence of an Avi-tag that is specifically recognized by the biotin ligase [15,16]. Using the Avi-tag approach requires an enzymatic step for the addition of biotin, a process that can be performed either *in vivo* or *in vitro*. However, its downside is that it can lead to aggregation and solubility issues as well, which is not surprising because of the hydrophobic nature of biotin moiety. Moreover, the effectiveness of *in vivo* (when target coexpressed with biotin ligase) biotinylation is very case specific.

Considering all the limitations associated with the current methods of immobilization of target proteins in phage display sorting experiments, there remains a need for alternate approaches that maintain the attributes of biotin associated tags while circumventing their shortcomings. Any alternate immobilization technique should retain the structural integrity and functional properties of the targets and not affect their expression and purification. Further, the tag should be reversible and have a high affinity to the immobilization substrate allowing enrichment of the desired clones over "background" binders, and thus improve the efficiency of phage display library sorting process. Based on extensive development and testing, we propose the use of an engineered N5A mutant of a 19-residue (RWKKNFIAVSAANRFKKIS) calmodulin binding peptide (CBP) as a C-terminal fusion tag that meets the demanding criteria required for a user-friendly and versatile alternative to biotin-based tags. We present here a comprehensive evaluation of the CBP using model systems drawn from examples that, in our hands, had proven problematic in the context of biotin-based tags. We show that the CBP tag does not adversely affect expression of the target to which it is fused, it has sufficient affinity to survive vigorous washing steps required during the sorting process, can be completely released from the immobilization substrate (calmodulin, CaM) by simple addition of ethylenediaminetetraacetic acid (EDTA) and it performs uniformly on virtually all targets compared to the target specific variability of the biotin-based tags.

Results

Target set

For model systems, we selected a diverse test set of antigen targets that ranged in size, stability and chemical makeup to evaluate the capabilities of the CBP immobilization tag for use in multiple phage display applications. Maltose binding protein (MBP) was chosen as a positive control since we have extensive experience with its properties and have generated numerous MBP-specific Fabs (*f*ragments *a*ntigen *b*inding) using a variety of tags allowing direct comparisons with other immobilization strategies. Our experience is that proteins with free cysteines are particularly challenging because they are prone to modification. Thus, we have included SETD7 (5 cysteine residues), HEF1 (4 cysteine residues) and GAP1 (single catalytic cysteine) in the test set. We note that an alternate immobilization method like chemical biotinylation, which uses a reagent (NHS-PEG_4-S-S-biotin) (*p*oly*e*thylene *g*lycol) that adds a biotin group cleavable by reducing agent, is not a feasible option because the samples always need to be preserved in a reducing environment containing DTT or TCEP. Some of the chosen targets (HEF1, bromo domain from *Drosophila melanogaster* and PGK from *Staphylococcus aureus*) are prone to aggregation after chemical biotinylation. Some targets in the set are small domains of multidomain proteins that are challenging to obtain binders for. The ligand binding domain of human estrogen receptor (ESRRA) and HEF1 are targets whose expression and solubility were significantly increased as CBP-fused constructs over the