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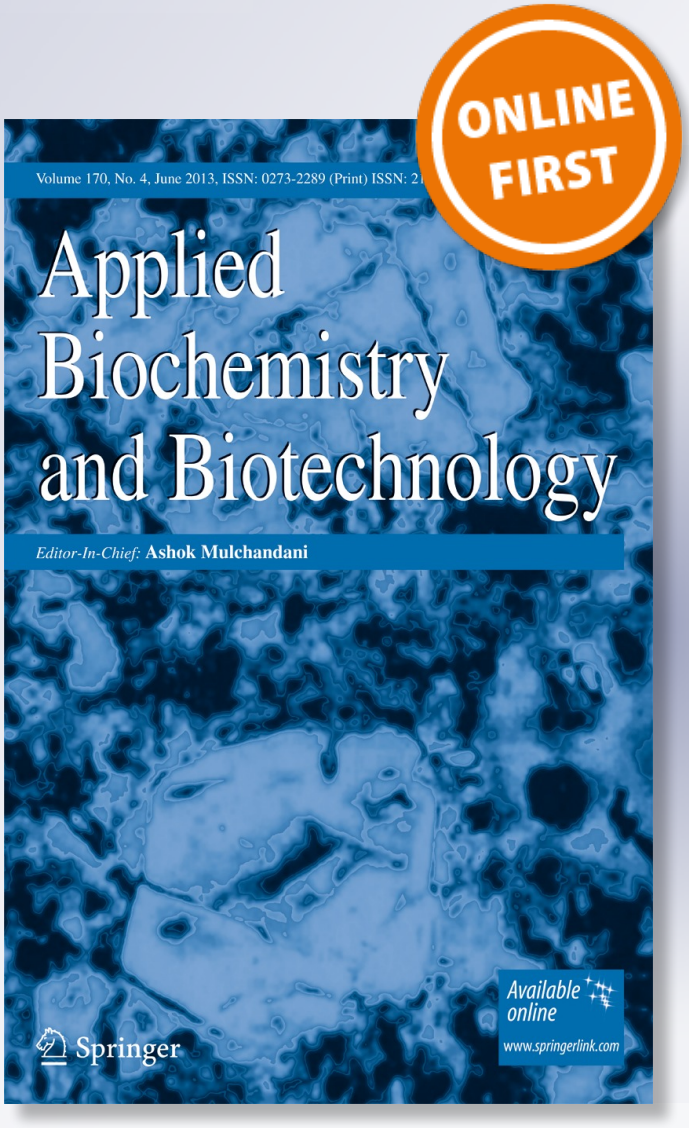

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Identification of Amino Acid Residues Responsible for the Binding to Anti-FLAG™ M2 Antibody Using a Phage Display Combinatorial Peptide Library

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Abstract FLAG, a short hydrophilic peptide consisting of eight amino acids (DYKDDDDK), has been widely used as a fusion tag for the purification and detection of a wide variety of recombinant proteins. One of the monoclonal antibodies against this peptide, anti-FLAG M2, recognises a FLAG peptide sequence at the N terminus, Met-N terminus, C terminus, or internal site of a fusion protein and has been extremely useful for the detection, identification, and purification of recombinant proteins. Nevertheless, detailed binding specificity of anti-FLAG M2 has yet to be determined. In the current study, a phage display combinatorial peptide library was used to determine that the motif DYKxxD encompasses the critical amino acid residues responsible for the binding of FLAG peptide to this antibody. This study demonstrates the utility of phage display technology and helps to elucidate the mode of action of this detection system.

Keywords FLAG · Monoclonal antibody · Phage display · Combinatorial peptide · Epitope tag · Anti-FLAG M2 · Mapping

Introduction

FLAG peptide was first developed 25 years ago as a small hydrophilic peptide of eight amino acids (AspTyrLysAspAspAspAspLys, DYKDDDDK) for antibody-mediated purification and identification of recombinant proteins [7]. The peptide was designed to consist of only eight amino acids so that it could be easily encoded in a single synthetic nucleotide while still allowing strong binding to an antibody. The five C-terminal amino acids (DDDDK) of the peptide represent the minimal enterokinase specificity site. Lysine (Lys, K) at position 3 of the peptide was chosen to ensure maximum hydrophilicity on a Hopp–Woods hydrophilicity scale [8].

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Aromatic amino acid tyrosine (Tyr, Y) was placed at position 2 to promote antibody interaction. Lastly, the N-terminal aspartic acid (Asp, D) was selected because it is a charged residue that can increase exposure of the Tyr to the antibody [7].

Since the development of FLAG peptide, several monoclonal antibodies specific to this peptide have been generated for immunopurification and various immunodetection purposes [4]. The first monoclonal antibody that was generated was the mouse monoclonal antibody clone 4E11 or M1, in which it was serendipitously discovered that Ca^{2+} was involved in the binding to the FLAG peptide in an affinity purification column [7]. As such, this FLAG monoclonal antibody can be used for calcium-mediated affinity purification of a wide variety of N-terminal fusion recombinant proteins from yeast, animal cells, and *Escherichia coli* [15]. In a calcium-independent manner, anti-FLAG M2, another mouse monoclonal antibody, can recognise a FLAG sequence at the N terminus, C terminus, Met-N terminus, and internal site of a fusion protein. Another mouse monoclonal anti-FLAG is clone M5, which only recognises N-terminal Met-FLAG fusion proteins from mammalian and *Drosophilae* expression systems. Moreover, rabbit anti-FLAG polyclonal antibody that can react with both N-terminal and C-terminal FLAG fusions is also commercially available. These antibodies have been widely used in molecular biology research [4].

To date, there have only been reports on the binding properties of anti-FLAG clone M1, which was determined to be specific for the first four amino acids (DYKD) of FLAG at the free N terminus of a fusion protein [15]. A study using a positional scanning combinatorial library approach determined that Asp at position 7 of FLAG is also required for complex formation [14]. However, analyses by surface plasmon resonance and enzyme-linked immunosorbent assay (ELISA) have provided conflicting results on the effect of calcium compared to studies using affinity chromatography [3]. Therefore, further investigation is necessary to fully understand the nature of the binding of this antibody. As for other anti-FLAG antibodies, no detailed specificity analyses of the binding properties have been reported.

Phage display technology is one of the most powerful methods to characterise binding specificity or the epitope of an antibody [9]. This method is based on affinity selection or "biopanning" of phage libraries displaying millions of peptides with randomised sequences for specific phage clones that display the interacting peptides [10]. Consensus motif can be deduced from multiple peptide sequences, and critical components of the protein binding domain [17] or antibody epitope can be defined [1, 12]. Since this approach is convenient and powerful, we attempted to employ this technique for the study on binding specificity of anti-FLAG M2, which is the most applicable FLAG antibody because it can bind to FLAG peptide located anywhere on the recombinant protein and be used for affinity purification in combination with peptide elution [2].

Materials and Methods

Biopanning with a Phage Display Peptide Library

Biopanning was performed as previously described [11]. One microgram of anti-FLAG M2 (catalog number S2BB7188, Sigma, USA) was used in each round of biopanning. Phage display of a 12-mers random peptide library (SUT12), which was prepared in our laboratory, was used in this experiment [11].

Phage ELISA

The binding activity of each individually selected phage clone was determined by ELISA as previously described [9]. Two hundred microliters of culture supernatant was added to each well of the ELISA plate (target and negative control) and incubated at room temperature for 2 h while rotating. Various control proteins that were used included bovine serum albumin (BSA), mouse anti-myc (BioRad, USA), purified rabbit polyclonal antibody, and purified recombinant enzymes from our laboratory, namely chitinase [16] and chitosanase [13]. Goat anti-mouse horseradish peroxidase (HRP) (BioRad, Canada) and anti-6xHis HRP (Thermo Scientific, USA) were used to confirm the presence of immobilised targets according to a previously published method [11]. The reactions were stopped by adding 1 % SDS, and the optical density was measured at 405 nm using an ELISA plate reader (Sunrise, TECAN, Austria).

Phage Peptide Sequence Analysis

Plasmids from positive phage clones were prepared as previously described [9]. DNA sequences were determined by automated DNA sequence analysis (Macrogen, Seoul, Korea) using the -96gIII primer (5′-CCC TCA TAG TTA GCG TAA CG-3′). Amino acid sequence analysis was done using Mac Vector software, as well as Basic Local Alignment Search Tool (BLAST) search (http://blast.ncbi.nlm.nih.gov/).

Results and Discussion

Affinity Selection of Anti-FLAG M2 Monoclonal Antibody

A combinatorial phage display random 12-mer peptide library was used for affinity selection against anti-FLAG M2 monoclonal antibody. After three rounds of biopanning, 2.5×10^7 pfu of phage clones were obtained. Thirteen clones were randomly selected and used to infect *E. coli* DH5αF′ for phage amplification. From these 13 clones, 6 clones (HF1, HF5, HF8, HF9, HF10, and HF12) showed specific binding to anti-FLAG M2. As shown in Fig. 1, all six selected phage clones could bind specifically to anti-FLAG M2, but not to recombinant chitosanase [13], chitinase [16], BSA, mouse anti-myc, or rabbit polyclonal antibody. To confirm the presence of immobilised targets at the bottoms of the wells of the ELISA plate, goat anti-mouse-HRP conjugate was used to detect mouse antibodies (anti-FLAG M2 and anti-myc), and anti-His-HRP was used to detect recombinant 6xHis-tagged chitosanase and chitinase. These results demonstrated the specific binding of selected phage clones to anti-FLAG M2. The procedure for biopanning was done following a standard method, which is normally used in our laboratory for the detection of peptide ligands of modular binding domains [17] or the epitope of a monoclonal antibody [12]. Affinity selection was performed for three rounds. Phage amplification was done only after the first round of panning, and no amplification was performed between the second and third round. Approximately half of the phage clones randomly selected from the third round of biopanning showed bona fide binding, indicating that the biopanning was successful. Based on our experience, an effective biopanning experiment will provide enough information to confer a consensus sequence. Furthermore, three rounds of biopanning is optimal for affinity selection since additional rounds will typically only lead to selection of phage clones that grow quickly, reducing the diversity of interacting

peptides. As such, only three rounds of biopanning was performed, and only 13 clones were selected for phage ELISA in the current study.

Amino Acid Sequence Analysis of Specific Peptides

Five phage clones that showed relatively high positive signals in ELISA were selected for automated DNA sequencing to determine their amino acid sequences. Clones HF5, HF8, HF9, HF10, and HF12 possessed different peptide sequences (Table 1). Remarkably, all of the selected peptides shared the same consensus sequence DYKxxD (x is any amino acid residue), suggesting that DYKxxD encompasses the critical amino acid residues responsible for the binding of FLAG peptide to anti-FLAG M2 antibody. This result is different from the binding specificity of anti-FLAG M1, which is specific for the first four amino acids (DYKD) and also requires the Asp at position 7 for complex formation [4].

Our results indicate that binding of mouse monoclonal anti-FLAG M2 is dependent on four amino acids in the FLAG peptide. Specifically, the first three amino acids of the FLAG peptide and the Asp at position 6 dictate the interaction. In addition, the amino acid adjacent to the C terminus of the DYKxxD motif is preferred to be highly hydrophilic (either basic or acidic), while the N-terminal amino acid is not restricted to any particular types. Differences

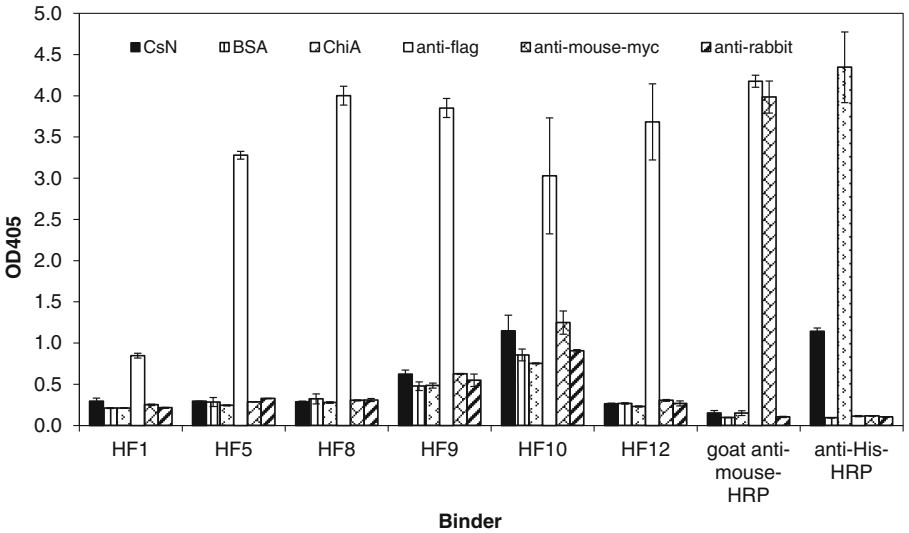

Fig. 1 Specific binding of phage to anti-FLAG M2. Six phage clones from the third round of biopanning were confirmed for their binding to anti-FLAG M2 monoclonal antibody by Phage ELISA. Duplicate wells of microtiter plate were coated with chitosanase (*CsN*), bovine serum albumin (*BSA*), chitinase A (*ChiA*), anti-FLAG M2 (*AF*), mouse monoclonal antibody against Myc epitope (*mouse anti-myc*), and rabbit polyclonal antibody (*rabbit antibody*) before incubation with phage clones HF1, HF5, HF8, HF9, HF10, HF12, HRP-conjugated goat anti-mouse monoclonal antibody (*goat anti-mouse HRP*), and HRP-conjugated anti-6xHis monoclonal antibody (*anti-His-HRP*), as described in the "Materials and Methods" section. Five phage clones that showed strong binding signal (OD_{405} more than 3.0) were subjected to DNA sequence analysis and their peptide sequences were shown in Table 1. Bindings of the phage displaying specific peptides were detected immunologically with an anti-phage antibody conjugated to horseradish peroxidase (*HRP*). ABTS was used as substrate for the detection by colorimetric reaction. Average OD values at 405 nm are shown

Table 1 The peptide sequences of selected phage clones

Phage clone	Peptide sequence
HF5	A L D *D Y K* A G *D* R G I
HF8	R Q G S W *D Y K* G A *D* R
HF9	A M A G P *D Y K* V A *D* K
HF10	Q *D Y K* H L *D* D L L Y A
HF12	Y R T M S *D Y K* L D *D* E
FLAG	*D Y K* D D *D* D K

Amino acid sequence alignment of anti-FLAG M2™ binding peptides, isolated from 12-mer M13 bacteriophage displayed combinatorial peptide library. The conserved amino acids are indicated in italics. The FLAG peptide sequence is shown in the bottom row. The random peptides are located at the N termini of minor phage coat proteins (pIII). The first 20 amino acids on the C-terminal side of the displayed random peptide are S R P S R T V E S C L A K S H T E N S F

in the binding characteristics between the M1 and M2 monoclonal antibodies against FLAG peptide help to explain the different requirement for Ca^{2+}. While the interaction of anti-FLAG M2 antibody is calcium-independent, that of the M1 antibody has been shown to be calcium-dependent in affinity chromatography experiments. Interestingly, amino acids at positions 3 and 4 can be nonpolar, acidic, or basic amino acids, indicating that maximum hydrophilicity is not necessary for specific binding of anti-FLAG M2. On the other hand, our results exemplify the role of an aromatic amino acid as a major factor in antigen–antibody interaction [5] as well as the two charged amino acids flanking the Tyr, which help promote the interaction of Tyr and the antibody binding site [6].

The specific peptides from this study were displayed on the N terminus of pIII capsid proteins, which are present in three to five copies at one end of the bacteriophage M13 [9]. In all selected phage clones, the DYKxxD motif was embedded in the middle of a 12-amino-acid-long peptide on the N terminus of the pIII coat protein. Typically, peptides isolated from pIII libraries have low micromolar (1–10 μM) dissociation constants (Kd) [9], which is consistent with the strong ELISA signals observed (Fig. 1).

BLAST search for the DYKxxD motif against all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF, excluding environmental samples from WGS projects, showed only

Table 2 BLAST search result against DYKxxD motif

Protein	Accession number	Aligned sequence[a]
Hypothetical protein, partial (uncultured bacterium)	AFJ95806.1	90 DYK-AD 95
AT3G55720-like protein, partial (*Capsella grandiflora*)	AEN82205.1	153 DYKS-D 158
DapA, partial (uncultured *Geobacter* sp.)	ABR01069.1	5 DYK-HD 10
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (*Tricholepis furcata*)	AFP23717.1	18 DYKTKD 23
Hypothetical protein VITISV_020305 (*Vitis vinifera*)	CAN76204.1	368 DYKESD 373

BLAST search was performed using NCBI BLASTP 2.2.27+ program against non-redundant database. A dash indicates blank space (no amino acid at this position)

[a] Numbers indicate amino acid positions of the protein

five hits, out of which only two hits showed six-amino-acid-long sequence, whereas the other four sequences are composed of five amino acids (Table 2). This provides a potential explanation for why this antibody yields very low background in various immunodetection experiments.

Conclusion

A phage display combinatorial peptide library was used to determine that the motif DYKxxD encompasses the critical amino acid residues that are responsible for the binding of FLAG peptide to anti-FLAG M2. The amino acid adjacent to the C terminus of the DYKxxD motif is preferred to be highly hydrophilic (either basic or acidic) followed by any kinds of amino acid sequences, while the N-terminal amino acids are not restricted to any particular types. This study demonstrates the effectiveness of phage display technology and provides insight into the mode of action of this detection system.

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