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Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5

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Summary

The FLAG® peptides DYKDDDDK and MDYKDDDDK are widely used affinity tags. Here we describe new variants of the FLAG peptides which, in direct ELISA, showed selective and differential binding to the commercially available anti-FLAG monoclonal antibodies M1, M2 and M5. Variants of the FLAG peptides were synthesized on polymer-grafted plastic pins, and in an ELISA incubated with mAbs M1, M2 and M5. Among the newly identified tag sequences are those that bind only one of the anti-FLAG mAbs and those that bind only two or all three of the anti-FLAG mAbs. Examples of new tag sequences are MDFKDDDDK (which binds mAb M5 and does not bind mAbs M1 and M2) and MDYKAFDNL (which binds mAb M2 and does not bind mAbs M1 and M5). The sensitivity in direct ELISA of some variants was increased, e.g. using mAb M2 it was found that replacing DDDDK in MDYKDDDDK by AFDNL increased the sensitivity in ELISA at least 10-fold. The activity of this peptide was studied in more detail. In different direct ELISAs, in which MDYKAFDNL was synthesized on polyethylene pins, coated onto polystyrene microtiter plates or onto nitrocellulose paper, the activity of this peptide was similar, i.e. increased at least 10-fold over that of MDYKDDDDK. Remarkably, in competitive ELISA the binding activity of soluble MDYKAFDNL was decreased 10-fold over those of soluble MDYKDDDDK or DYKDDDDK. The results seem to suggest that, in solution, the conformation of MDYKAFDNL is more 'unstructured' compared to its conformation when coated or linked to a carrier. We postulate that the newly described tag sequences may be used as affinity tags to separately detect, quantify and purify multiple co-expressed proteins and/or subunits.

Introduction

Detection, quantification and purification of recombinant proteins is facilitated using affinity tags that are genetically fused to the gene of interest [1,2]. Examples of widely applicated affinity tags include the polyhistidine tag [3] and short epitope tags such as the c-myc peptide and the FLAG peptides [4–6]. Affinity technologies using tag sequences with selective recognition properties have stimulated various aspects of separation and analysis techniques [2]. Here new FLAG sequences are described and their potential as new affinity tags is discussed.

The original FLAG sequences DYKDDDDK and MDYKDDDDK are affinity tags that are short and, when required, enzymatically removable with enterokinase [4,5,7]. Using the commercially available mAbs M1, M2 and M5, many proteins genetically fused to the FLAG peptides have been detected, quantified and purified [4,8]. Often the FLAG peptide DYKDDDDK has been shown not to interfere with important structural and functional properties of the expressed proteins, an important feature when enterokinase is not or cannot be used [1,8].

Specific properties of mAb M1 include Ca^{2+}-dependent binding, which can be used for Ca^{2+}-mediated affinity

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Abbreviations: ABTS, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate; ELISA, enzyme-linked immunoabsorbent assay; GDA, glutardialdehyde; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; rampo, rabbit-anti-mouse peroxidase; SDS, sodium dodecylsulfate.

purification, and binding that is three to four orders of magnitude better when the amino-terminal α-amino group of DYKDDDDK is freely accessible [4,5,9]. Its dependence on a free α-amino group requires that DYKDDDDK follows a cleavage site, such as a signal sequence. This condition has been shown to be compatible with efficient processing in bacteria [8]. However, when present at the very amino terminus of nonsecreted proteins, the amino-terminal methionine is not cleaved (K. Proba et al., unpublished results). This is expected from the preferences of amino-terminal methionine removal [10–12]. Therefore, M1 is not useful for cytoplasmic proteins or for in vitro translation.

MAbs M2 and M5 are Ca^{2+}-independent binders and can be used at the amino terminus of unprocessed proteins (behind methionine) or within or at the end of protein sequences, since their binding does not depend on an amino-terminal α-amino group. MAbs M1 and M2 have been recommended to detect DYKDDDDK and mAb M5 has been recommended to detect the longer FLAG peptide MDYKDDDDK, but this epitope sequence had so far not been studied in more detail.

Recently, for mAb M1 variants of DYKDDDDK were described that had 6–100-fold higher activities than the original FLAG peptide [8,9]. Here additional new variants of DYKDDDDK and new variants of MDYKDDDDK are described. These include sequences with higher activities and/or differential recognition properties for mAbs M1, M2 and/or M5. Potential applications of these new variants are discussed.

Materials and Methods

Monoclonal antibodies

MAbs M1, M2 and M5 are murine IgG monoclonal antibodies that were purchased from the Eastman Kodak Company (New Haven, CT, U.S.A.). MAbs M1 and M2 were raised against DYKDDDDK and mAb M5 was raised against MDYKDDDDK. The binding of mAb M1 to DYKDDDDK is Ca^{2+}-dependent, whereas the binding of mAbs M2 and M5 to DYKDDDDK and MDYKDDDDK, respectively, is not. $CaCl_2 \cdot 2H_2O$ was purchased from Merck (Amsterdam, The Netherlands, no. 2382). The amino acid positions in the FLAG peptides are referred to as α-amino or M0, D1, Y2, K3, D4, D5, D6, D7 and K8.

Peptide synthesis

Support-bound peptides representing the FLAG peptides and many variants of the FLAG peptides were synthesized as described previously [13]. The peptides were synthesized on polyethylene pins that are part of holders that fit into 96-well microtiter plates (Greiner, Alphen aan den Rijn, The Netherlands). The variants of the FLAG peptides included single and multiple replacements and semirandom variants with the consensus sequence DYKXXXXX (where X is a randomly selected residue from the 20 natural L-amino acids). The 50 random XXXXX sequences were generated with a random generator programmed in Quick Basic, which runs on a 486 DX2 (66 MHz) computer system. In these sequences the frequency of each residue is approximately 5%. All variants carry a free amino-terminal α-amino group.

To compare the results obtained in the pin ELISA with 'standard' microtiter ELISAs, the peptides DYKDDDDK-NH_2, MDYKDDDDK-NH_2, MDFKDDDDK-NH_2 and MDYKAFDNL-NH_2 were synthesized and purified by HPLC (to > 90% purity) as described previously [14]. These four peptides also carry a free amino-terminal α-amino group.

Direct ELISA

The peptides synthesized on pins or coated onto microtiter plates were incubated with antibody concentrations ranging from 0.001 to 5 μg/ml. With mAb M1, all incubations were performed in the presence or absence of 1 mM $CaCl_2$; this is denoted in the text as mAb M1 ($+Ca^{2+}$) and mAb M1 ($-Ca^{2+}$).

The coating of the microtiter plates was done using glutardialdehyde (GDA): 100 μl of a solution of 0.2% GDA in 0.1 M phosphate buffer, pH 5.0, was added to 96-well plates, incubated for 3 h at 20 °C while shaking and washed twice with 0.1 M phosphate buffer, pH 8.0. Then 100 μl of a solution of 10 μM peptide in 0.1 M phosphate buffer, pH 8.0, was added to the GDA-coated plates. After an incubation period of 4 h at 37 °C while shaking, the plates were washed twice with 0.1 M phosphate buffer, pH 8.0. Unreacted aldehyde groups were 'saturated' with medium A (see below).

For the ELISA on microtiter plates the mAbs were dissolved in medium A, while for the ELISA on pins the mAbs were dissolved in medium B. Medium A is a phosphate buffer (pH 7.2) to which were added 4% horse serum, 1% Tween-80 and 2.1% NaCl. Medium B is a phosphate buffer (pH 7.2) to which were added 5% ovalbumin, 5% horse serum and 1% Tween-80. These additions were used to block nonspecific binding. In both ELISAs the mAbs were incubated overnight at 4 °C. After washing, the peptides were incubated with rabbit-anti-mouse peroxidase (rampo) (1/1000) (Dako, Glostrup, Denmark) for 1 h at 25 °C and subsequently, after washing, with the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 μl/ml 3% H_2O_2. After 1 h the absorbance (at 405 nm) was measured with a multiscan ELISA plate reader (Titertek, Flow Laboratories, McLean, VA, U.S.A.). After each assay the pin peptides were cleaned in sodium dodecylsulfate (SDS) and mercaptoethanol-containing solution and re-used.

Competitive ELISA

The competitive ELISA is a modification of the direct

TABLE 1
ALANINE AND HOMOLOGY SCANNING OF DYKDDDDK

No.	Sequence	mAb			
	1 2 3 4 5 6 7 8	M1+	M1–	M2	M5
1	D Y K D D D D K	2.1	1	3	1.9
2	A Y K D D D D K	0.1	0.1	0.3	0.5
3	D A K D D D D K	0.1	0.1	0.1	0.2
4	D Y A D D D D K	0.1	0.1	0.1	2
5	D Y K A D D D K	1.6	0.7	0.5	0.2
6	D Y K D A D D K	2.9	1.3	2.8	0.2
7	D Y K D D A D K	1.8	1.4	0.9	1
8	D Y K D D D A K	1.7	0.3	1.8	0.5
9	D Y K D D D D A	1.6	0.8	2.7	1.8
10	E Y K D D D D K	1.3	0.3	0.5	0.3
11	D F K D D D D K	0.1	0.1	0.2	2.4
12	D Y R D D D D K	0.7	0.2	0.2	2.3
13	D Y K E D D D K	1.9	1	2.2	1.4
14	D Y K D E D D K	2	0.9	2.8	0.9
15	D Y K D D E D K	2	1	2.4	1.8
16	D Y K D D D E K	1.7	0.5	2.7	1.6
17	D Y K D D D D R	2.4	1	3.2	1.9

Values correspond to O.D. (at 405 nm); mAb M1+: 1 μg/ml (1 mM $CaCl_2$); mAb M1–: 1 μg/ml (no $CaCl_2$); mAb M2: 1 μg/ml; mAb M5: 5 μg/ml.

microtiter ELISA described above. The peptides DYKDDDDK-NH_2, MDYKDDDDK-NH_2 and MDYKAFDNL-NH_2 were coated onto microtiter plates and incubated with mAb M2 (0.15 μg/ml medium A) in the presence of varying concentrations of the different synthetic peptides. The concentration of the dissolved peptides was checked using amino acid analysis. The pin ELISA was not used as a competitive pin ELISA, because peptides on pins are not well defined since they cannot be purified. Two independent experiments were done, each in duplicate. The variation between the two experiments was < 25%. Sigmoidal binding curves for each peptide were obtained using Excel 4.0 (Microsoft software). The IC_{50} of each peptide (the concentration necessary to inhibit 50% of the mAb binding to the coated peptide) was then determined.

Dotting immuno assay

The peptides DYKDDDDK-NH_2, MDYKDDDDK-NH_2 and MDYKAFDNL-NH_2 were dissolved in distilled water and dotted onto nitrocellulose paper (0.45 μm pore size, Schleicher and Schuell, Dassel, Germany) ranging from 500 ng/μl/dot to 16 ng/μl/dot (each dot was approximately 0.03 cm^2). Since small synthetic peptides do not adhere easily to nitrocellulose paper, the strips were incubated for 60 min at 110 °C according to Li et al. [15]. Subsequently, the strips were incubated with anti-FLAG M2 (1 μg/ml medium A) for 1 h at 25 °C, washed three times with medium A, and then incubated with rampo (1/1000; Dako) for 1 h at 25 °C. After washing, the strips were treated with 3,3-diaminobenzidine (DAB) (Sigma, St. Louis, MO, U.S.A.) containing 1% cobalt chloride/1% nickel chloride and 2 μl/ml 3% H_2O_2 as described by DeBlas et al. [16]. The color reactions of the nitrocellulose strips were translated into grey values using an image processing system composed of an image scanner (Hewlett-Packard Scanjet IIcx), Aldus Photostyler 2.0 running on a Pentium Compaq Presario 9220.

Results

Replacing individual residues in DYKDDDDK with alanine or a structurally related residue (D by E, Y by F and K by R) showed that, in the presence of Ca^{2+}, residues D1, Y2 and K3 are most critical for binding mAb M1. Without Ca^{2+}, however, the binding to all these single-position variants is reduced and D1, Y2 and K3 and to a lesser extent D4 and D7 are most critical for binding mAb M1 (Table 1, nos. 1–17). For mAb M2, residues Y2, K3 and to a lesser extent D1 and D6 are most critical for binding (Table 1, nos. 1–17).

Although mAb M5 was raised against MDYKDDDDK, it was found that M5 also binds DYKDDDDK (Table 1, no. 1). For DYKDDDDK it was found that Y2, D4 and D5 and to a lesser extent D1 and D7 are most critical for binding M5 (Table 1, nos. 2–17; Table 2,

TABLE 2
MULTIPLE REPLACEMENT ANALYSIS OF DYKDDDDK

No.	Sequence	mAb			
	1 2 3 4 5 6 7 8	M1+	M1–	M2	M5
1	D F R D D D D K	0.1	0.1	0.1	2.9
2	D F R D D D D R	0.1	0.1	0.1	2.5
3	D F K E E E E K	0.1	0.1	0.1	2.6
4	E F K D D D D K	0.1	0.1	0.1	0.3
5	D Y K A A A A K	1.4	1.4	0.1	0.1
6	D Y K D A A A K	2.9	2	0.1	0.1
7	D Y K D D A A K	2.6	2.6	0.6	0.2
8	D Y K A A A A A	1.6	1.6	0.1	0.1
9	D Y K D A A A A	2.1	2.1	0.2	0.1
10	D Y K D D A A A	2.2	1.6	0.6	0.6
11	D Y K D D D A A	2.2	1.2	2.7	1.1
12	D Y K K K K K K	2.6	2	0.1	0.1
13	D Y K D K K K K	1.9	2	0.1	0.1
14	D Y K D D K K K	2.3	2	0.2	0.1
15	D Y K D D D K K	2	1.7	2.8	0.2
16	D Y K D A A K K	2.8	2.4	0.1	0.1
17	D Y K E A A K K	1.8	2.4	0.1	0.1
18	D Y K E A K K K	1.3	1.5	0.1	0.1
19	D Y K E K K K K	1.3	1.6	0.1	0.1
20	D Y K R T D W C	2.6	0.9	0.9	0.1
21	D Y K V V D D R	2.1	1.9	1.4	0.1
22	D Y K H L D N S	1.6	0.9	1.5	0.1
23	D Y K A F D N L	1.8	1.3	2.1	0.1
24	D Y K X K X *	1.9–2.6	1.6–3	0.1–0.2	0.1
25	D Y K E K X X X D **	1.8–2.8	1.6–3.1	0.1	0.1

Values correspond to O.D. (at 405 nm); mAb M1+: 1 μg/ml (1 mM $CaCl_2$); mAb M1–: 1 μg/ml (no $CaCl_2$); mAb M2: 1 μg/ml; mAb M5: 5 μg/ml; *, consensus sequences represent sequences of Tables 1 and 2 from Ref. 9.

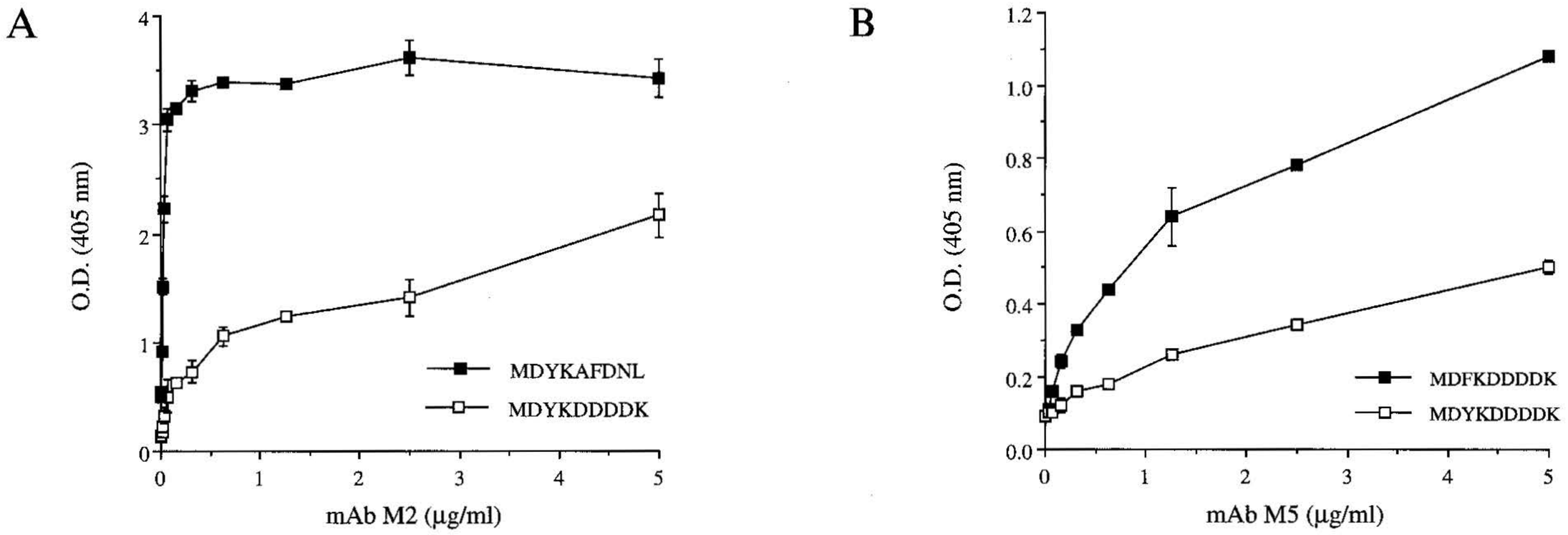

Fig. 1. Direct ELISA with (A) mAb M2 and (B) mAb M5 on peptides coated onto microtiter plates.

no. 15). Remarkably, in MDYKDDDDK residue D7 seems not to be critical for binding M5 (cf. Table 1, no. 8 and Table 2, no. 15 with Table 3, nos. 5 and 10).

The role of the negatively charged Ds in the 'DDDD-K'-region in binding mAbs M1, M2 and M5 was investigated using various combinations of alanine and/or lysine replacements. Most of these multiple replacements did not result in a dramatic decrease of binding to mAb M1 ($+Ca^{2+}$) (Table 2, 5–15). Binding to M1 ($-Ca^{2+}$) was often increased, i.e. the binding was often no longer calcium dependent (Table 2, 5–15). Binding to mAbs M2 and M5 was often decreased (Table 2, 5–15).

The group of previously identified FLAG peptide variants having the consensus sequences DYKXKX and DYKEKXXXDD (X = various residues allowed; cf. Ref. 9) were equally well recognized by M1 ($+Ca^{2+}$) and M1 ($-Ca^{2+}$) and were not recognized by mAbs M2 and M5 (Table 2, nos. 24 and 25).

Of a group of 50 semi-random peptides with the consensus sequence DYKXXXXX (X = a randomly chosen residue), most were recognized by M1 ($+Ca^{2+}$) and/or M1 ($-Ca^{2+}$). Only four of these peptides were recognized by M2 and none were recognized by M5 (Table 4).

The sensitivity in ELISA of the original FLAG peptide for M1 ($-Ca^{2+}$) was decreased compared to that for M1 ($+Ca^{2+}$) (Table 1, no. 1). Most of the variants gave a similar difference in sensitivity. However, peptides for which the sensitivity in ELISA for mAb M1 did not depend on the presence of Ca^{2+} are some of the random peptides (Table 4, nos. 17, 25 etc.), the DYKXKX and DYKEKXXXDD consensus peptides (Table 2, nos. 24 and 25) and most of the multiple alanine/lysine replacement variants (Table 2, nos. 5–15).

Variants of the second FLAG peptide MDYKDDDDK, containing an additional amino-terminal methionine, were also synthesized and tested (Table 3). Although MDYKDDDDK was not recognized by mAb M1, some of the variants were, albeit with lower binding activity (Table 3, nos. 8 and 16–18). The latter variants were not recognized by mAbs M2 and M5. Other variants of MDYKDDDDK were only recognized by M2 (Table 3, no. 14) or were only recognized by M2 and M5 (Table 3, no. 6, etc.).

The peptides DYKDDDDK, MDYKDDDDK, MDFKDDDDK and MDYKAFDNL were also synthesized, purified and used in standard microtiter ELISA tests, in dotting immuno assays and in competitive ELISA tests.

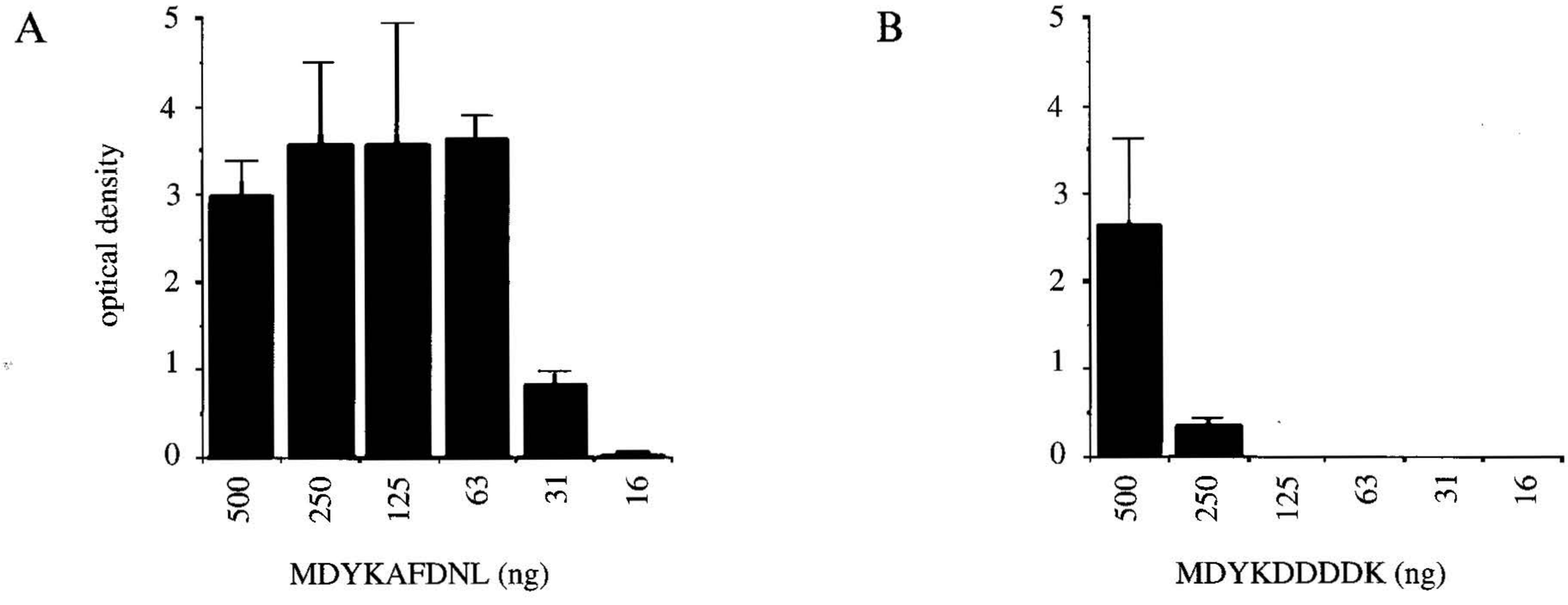

Fig. 2. Dotting immuno-assay with mAb M2. The peptides were coated from 500 to 16 ng/μl/dot. Each dot was approximately 0.03 cm^2 in size. The antibody concentration was 0.15 μg/ml. The optical density is shown in arbitrary units.

TABLE 3
REPLACEMENT ANALYSIS OF MDYKDDDDK

No.	Sequence									mAb			
	0	1	2	3	4	5	6	7	8	M1+	M1−	M2	M5
1	M	D	Y	K	D	D	D	D	K	0.1	0.1	1.6	2.3
2	M	D	Y	K	A	D	D	D	K	0.1	0.1	0.2	0.7
3	M	D	Y	K	D	A	D	D	K	0.1	0.1	0.9	0.4
4	M	D	Y	K	D	D	A	D	K	0.1	0.1	0.2	2.2
5	M	D	Y	K	D	D	D	A	K	0.1	0.1	1.4	3.1
6	M	D	Y	K	D	D	D	D	A	0.1	0.1	1.7	2.6
7	M	D	Y	K	K	D	D	D	K	0.2	0.2	1.5	0.6
8	M	D	Y	K	D	K	D	D	K	1	0.9	2.8	1.1
9	M	D	Y	K	D	D	K	D	K	0.3	0.3	0.2	2.4
10	M	D	Y	K	D	D	D	K	K	0.2	0.2	2.2	3
11	M	D	F	K	D	D	D	D	K	0.1	0.1	0.1	3.5
12	M	D	Y	R	D	D	D	D	K	0.1	0.1	0.1	3.1
13	M	D	F	R	D	D	D	D	K	0.1	0.1	0.1	3.2
14	M	D	Y	K	A	F	D	N	L	0.1	0.1	2.6	0.1
15	M	D	Y	K	D	D	D	A	A	0.1	0.1	0.8	1.9
16	M	D	Y	K	D	A	A	A	K	1	0.1	0.1	0.1
17	M	D	Y	K	D	A	A	K	K	1.1	0.4	0.1	0.1
18	M	D	Y	K	D	H	M	R	L	0.8	0.1	0.1	0.1

Values correspond to O.D. (at 405 nm); mAb M1+: 1 μg/ml (1 mM $CaCl_2$); mAb M1−: 1 μg/ml (no $CaCl_2$); mAb M2: 1 μg/ml; mAb M5: 5 μg/ml.

With mAb M2 the sensitivity of MDYKAFDNL in the microtiter ELISA, the pin ELISA and in the dotting immuno assay was increased approximately 10-fold compared to that of MDYKDDDDK (Figs. 1A and 2). With mAb M5 the sensitivity in ELISA of MDFKDDDDK (Y2 replaced by F) was increased approximately 10-fold compared to that of MDYKDDDDK (Fig. 1B). With the same sequences, similar results were obtained in the pin ELISA, i.e. a 10-fold difference in sensitivity was also observed (for example, cf. Table 3, no. 1 with no. 11).

A competitive ELISA, in which the peptides DYKDDDDK, MDYKDDDDK and MDYKAFDNL were coated onto microtiter plates and competed for binding mAb M2 with soluble DYKDDDDK, MDYKDDDDK or MDYKAFDNL, showed that the binding activity of peptides linked to a carrier not necessarily correlates with the binding activity of the peptides in solution (Table 5). Regardless of which peptide was coated, the soluble peptides DYKDDDDK and MDYKDDDDK had IC_{50}'s that were approximately 10-fold lower than the IC_{50} obtained with soluble MDYKAFDNL. Furthermore, when MDYKAFDNL was coated, all IC_{50} values were approximately 50–100-fold higher than when DYKDDDDK or MDYKDDDDK were coated (Table 5).

Discussion

Identification of critical residues

In the pin ELISA it was found that the free α-amino group and residues D1, Y2 and K3 are most critical for binding mAb M1 (+Ca^{2+}). This indicates that DYK, with a free amino group, is the dominant epitope for M1. In the absence of Ca^{2+}, all signals were weaker and the pres-

TABLE 4
SEMI-RANDOM REPLACEMENT ANALYSIS OF DYKDDDDK

No.	Sequence								mAb			
	1	2	3	4	5	6	7	8	M1+	M1−	M2	M5
1	D	Y	K	T	Y	Q	R	T	2.4	2.2	0.1	0.1
2	D	Y	K	V	W	M	D	D	2.1	0.7	0.3	0.1
3	D	Y	K	Q	S	C	F	T	2.4	1.7	0.1	0.1
4	D	Y	K	Q	T	A	L	N	2.6	1.7	0.1	0.1
5	D	Y	K	R	T	D	W	C	2.6	0.9	0.9	0.1
6	D	Y	K	V	V	D	D	R	2.1	1.9	1.4	0.1
7	D	Y	K	D	W	H	Q	G	1.6	0.4	0.2	0.1
8	D	Y	K	V	M	E	V	D	1.8	1.3	0.2	0.1
9	D	Y	K	W	V	Y	G	A	1.8	1.1	0.1	0.1
10	D	Y	K	S	Q	A	R	P	1.1	1.4	0.1	0.1
11	D	Y	K	W	E	Q	L	P	1.8	1.3	0.3	0.1
12	D	Y	K	W	G	M	Y	K	1.9	2	0.1	0.1
13	D	Y	K	K	M	A	H	Q	1.7	2.3	0.1	0.1
14	D	Y	K	V	M	F	W	L	1.9	1	0.1	0.1
15	D	Y	K	A	K	V	K	C	2.7	2.1	0.1	0.1
16	D	Y	K	V	C	I	W	N	2.1	0.6	0.1	0.1
17	D	Y	K	E	Q	K	A	H	2.4	2.6	0.1	0.1
18	D	Y	K	G	W	V	T	K	1.8	1.6	0.1	0.1
19	D	Y	K	Q	G	F	W	I	2.5	1.8	0.1	0.1
20	D	Y	K	Q	I	K	E	S	1.9	1.5	0.1	0.1
21	D	Y	K	A	D	L	W	I	2.2	1.9	0.1	0.1
22	D	Y	K	P	L	M	D	E	0.7	0.3	0.4	0.1
23	D	Y	K	Q	V	Q	V	P	1.7	2.2	0.1	0.1
24	D	Y	K	E	A	K	K	K	1.3	1.5	0.1	0.1
25	D	Y	K	D	H	M	R	L	2.1	2.6	0.2	0.1
26	D	Y	K	F	H	N	T	A	2	2.1	0.6	0.1
27	D	Y	K	T	M	Q	H	L	2.1	0.7	0.1	0.1
28	D	Y	K	G	T	F	Y	G	2.2	1.9	0.1	0.1
29	D	Y	K	C	L	C	L	P	2.3	0.6	0.1	0.1
30	D	Y	K	C	D	H	H	M	1.8	0.7	0.1	0.1
31	D	Y	K	I	V	N	A	H	1.4	1.6	0.1	0.1
32	D	Y	K	T	D	Q	Y	L	2.1	1.6	0.1	0.1
33	D	Y	K	H	L	D	N	S	1.6	0.9	1.5	0.1
34	D	Y	K	I	C	V	P	Y	1.8	0.9	0.1	0.1
35	D	Y	K	E	F	V	P	G	2.1	2.2	0.1	0.1
36	D	Y	K	T	I	Y	P	V	2	2.4	0.1	0.1
37	D	Y	K	M	L	W	Y	N	2.1	1.8	0.1	0.1
38	D	Y	K	H	N	N	Y	N	1.8	0.4	0.1	0.1
39	D	Y	K	K	M	Y	N	R	2.4	1.7	0.1	0.1
40	D	Y	K	W	F	P	M	G	2.2	0.3	0.1	0.1
41	D	Y	K	E	R	R	H	H	2.6	1.5	0.1	0.1
42	D	Y	K	V	L	N	I	L	2.5	2.2	0.1	0.1
43	D	Y	K	S	R	Y	S	S	2.1	2.1	0.1	0.1
44	D	Y	K	D	M	Y	C	A	1.9	0.7	0.1	0.1
45	D	Y	K	A	F	D	N	L	1.8	1.3	2.1	0.1
46	D	Y	K	D	H	N	Y	G	1.8	0.4	0.3	0.1
47	D	Y	K	V	C	W	E	G	1.8	0.8	0.3	0.1
48	D	Y	K	P	L	L	S	E	0.8	0.5	0.1	0.1
49	D	Y	K	A	G	F	W	R	2.2	2.5	0.1	0.1
50	D	Y	K	P	F	W	V	R	1.5	0.4	0.1	0.1

Values correspond to O.D. (at 405 nm); mAb M1+: 1 μg/ml (1 mM $CaCl_2$); mAb M1−: 1 μg/ml (no $CaCl_2$); mAb M2: 1 μg/ml; mAb M5: 5 μg/ml.

TABLE 5
COMPETITIVE ELISA WITH mAb M2

No.	Peptide coated onto microtiter plate (1 nmol/100 µl/well/4 h)	Peptides in solution		
		DYKDDDDK	MDYKDDDDK	MDYKAFDNL
1	D Y K D D D D K	0.08 ± 0.02	0.06 ± 0.01	0.65 ± 0.06
2	M D Y K D D D D K	0.15 ± 0.02	0.22 ± 0.07	1.83 ± 0.51
3	M D Y K A F D N L	9.48 ± 0.23	8.00 ± 0.89	75.0 ± 5.65

Values correspond to IC_{50} (µM); the concentration mAb M2 was 0.15 µg/ml.

ence of D7, and to a lesser extent D4, was also required. These findings are in good agreement with previous studies in which synthetic and recombinant peptides were used. A free α-amino group, residues D1, Y2 and K3 and, in the absence of Ca^{2+}, residues D4 and D7, were found to be most critical for binding mAb M1 [5,8,9].

For mAb M2, it was found that residues Y2 and K3 and to a lesser extent D1 and D6 are most critical. These findings are also in good agreement with previous studies, in which the phage-display technology and NMR spectroscopy were used. Residues Y2 and K3 and to a lesser extent D1 and D6 were found to be most critical for binding mAb M2 [17–19].

For mAb M5, it was found that residues D1, D4 and D5 and to a lesser extent Y2 are most critical. To our knowledge, phage-display or biophysical studies with mAb M5 have not been done. Consensus sequences for recognition of mAbs M1, M2 and M5 are shown in Fig. 3.

The role of Ca^{2+} in binding mAb M1

Recently, the role of Ca^{2+} in the binding of mAb M1 to DYKDDDDK has been investigated in detail. Residues D1, D4 and especially D7 seem to be strongly involved in Ca^{2+} binding [5,9]. We obtained a similar result (cf. Table 1). It has been postulated that, in the absence of Ca^{2+}, a positively charged residue such as K in DYKAKE 'replaces' the Ca^{2+} and as a result makes the binding of these peptides to mAb M1 calcium independent [9]. This possibility is supported by our results obtained with the 50 semi-random peptides (Table 4). Many of the semi-random peptides that bind mAb M1 in a calcium-independent manner contain a positively charged residue in the 'DDDDK' region (Table 4, nos. 1, 6, 12, 13, 17, 24, 25, 43 and 49). However, not all peptides that bind mAb M1 in a Ca^{2+}-independent manner contain a positively charged residue in the 'DDDDK' region (Table 4, nos. 23, 35, 36 etc. and Table 2, nos. 8 and 9). The dependence on Ca^{2+} of the binding of mAb M1 to the original FLAG peptide may indicate that the antibody recognizes a particular conformation which is populated only in the presence of Ca^{2+}. The results discussed above suggest that in the absence of the charged 'DDDD' cluster, this 'productive' conformation may be sufficiently populated as well. It is also possible that some sort of electrostatic repulsion exists between mAb M1 and DYKDDDDK. The addition of Ca^{2+} or the replacement of the 'DDDD' cluster would neutralize this electrostatic repulsion.

Differential and selective binding to tag sequences

It can be envisaged that tag sequences that bind only one or two of the anti-FLAG mAbs may be used to discriminate between cross-reacting proteins [19] or to separately detect, quantify and purify two or three different co-expressed proteins and/or subunits. For example, in a co-expression experiment of two proteins, one tagged with a FLAG peptide that is recognized by mAbs M2 and M5 (but not by M1) and the other tagged with a FLAG peptide that is recognized by mAbs M2 and M1 (but not by M5) could be first detected together, quantified and purified with mAb M2 and secondly separately detected and quantified with mAbs M1 and M5. Another interesting application is in the study of protein processing. If the DYKD sequence follows a signal cleavage site, e.g. for bacterial transport [8], only the product can be recognized by mAb M1. It may be useful to define other sequences that can selectively mark the precursor, to follow both species on a Western blot.

Before discussing such tag sequences, we must address the question whether the binding activity of the tag sequences in the pin ELISA corresponds to the binding activity in detection and separation techniques such as Western blots and affinity purification. We believe this to be the case. First, the results obtained with the pin ELISA, the microtiter ELISA and the dotting immuno assay do not differ dramatically (Tables 1–4 and Figs. 1 and 2). Second, the critical residues that were identified using the

anti-FLAG M1 (+$CaCl_2$)	H_2N-DYK.....
anti-FLAG M1 (–$CaCl_2$)	H_2N-DYKD..D.
anti-FLAG M2	. -DYK..D..
anti-FLAG M5	. -DY..DD..

Fig. 3. Critical residues involved in binding mAbs M1, M2 and M5. Dots indicate noncritical positions.

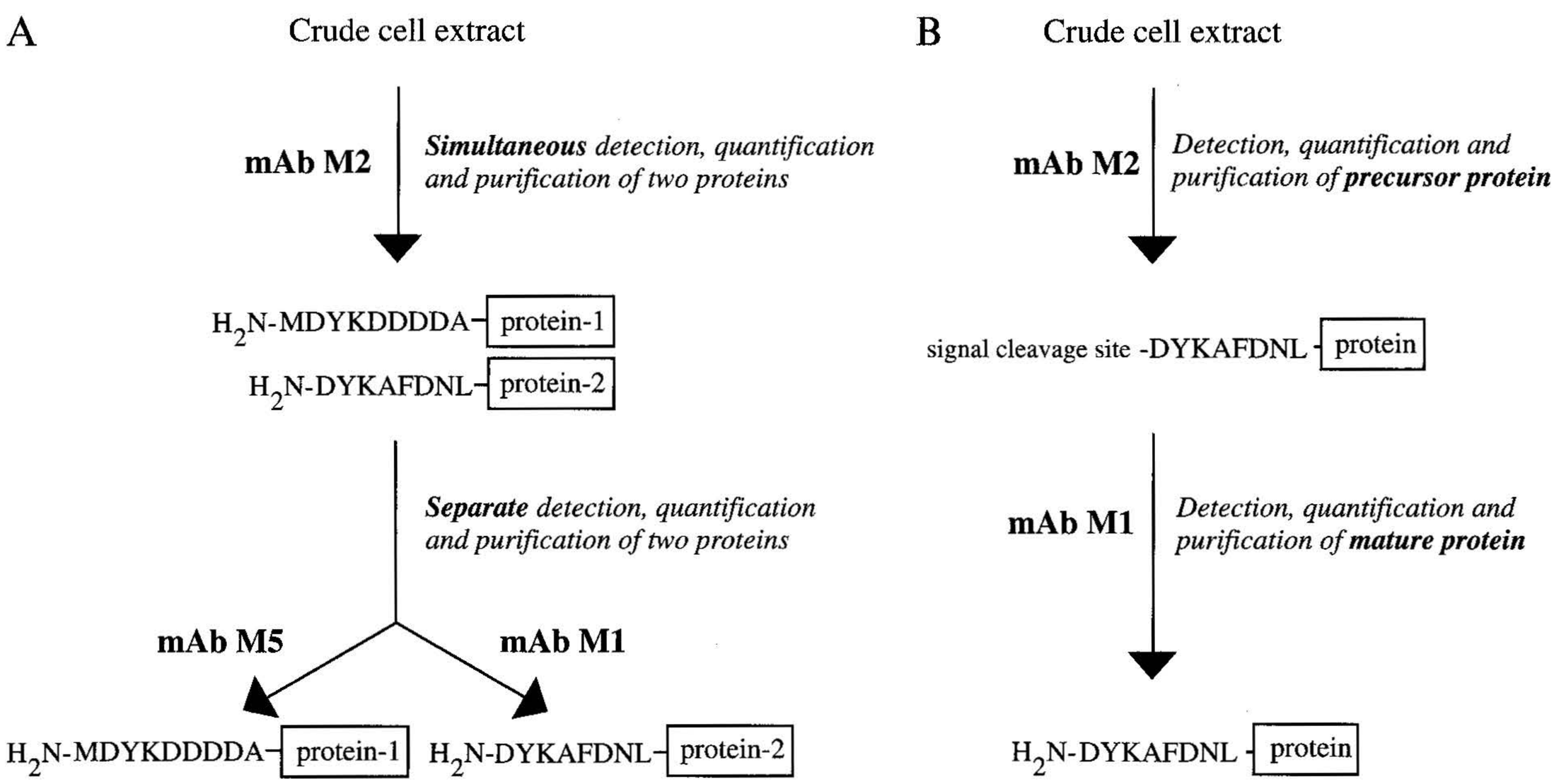

Fig. 4. Examples of putative applications of the new FLAG peptide variants. (A) Simultaneous and separate detection, quantification and purification of two expressed proteins or subunits; (B) separate detection, quantification and purification of the precursor and the mature form of a particular protein. H_2N-MDYKDDDDA is only recognized by mAbs M2 and M5; H_2N-DYKAFDNL is only recognized by mAbs M2 and M1; DYKAFDNL (without amino-terminal α-amino group) is only recognized by mAb M2; H_2N-DYKAFDNL is only recognized by mAbs M1 and M2 (cf. Tables 1–4).

pin ELISA correspond well with those identified using various other techniques [5,8,9,17–19]. Whatever technique was used, the presence of the right combination of critical residues seems to determine binding to the anti-FLAG mAbs. Thus, from these data obtained with synthetic peptides it seems possible to suggest the newly described tag sequences as selective affinity tags.

Peptides that bind mAb M1 but do not bind mAbs M2 and M5 include DYKDAAAK, DYKKKKKK (Table 2, nos. 6 and 12) and the DYKXKX and DYKEKXXXDD consensus sequences (Table 2, nos. 24 and 25). These peptides do not bind M2 or M5, probably because residues D4, D5 and/or D6 have been replaced (cf. Fig. 3).

Peptides that bind mAb M2 but do not bind mAbs M1 and M5 were initially not found. However, when 50 semi-random peptides with the consensus sequence DYKXXX-XX were synthesized (X = various residues allowed), it was found that only 4 of these 50 peptides bind mAb M2. These four peptides have the consensus sequence DYKX-XDXX (X = various residues allowed) (Table 4, nos. 5, 6, 33 and 45). It was found that lengthening of one of these peptides to MDYKAFDNL resulted in a peptide that only binds mAb M2 (Table 3, no. 14). An important advantage of this peptide is the fact that it starts with the translational start residue methionine, i.e., there is no additional need to fuse a specific cleavage site to the amino terminus of the FLAG sequence (cf. Refs. 10–12). The peptide MDYKAFDNL does not bind mAbs M1 or M5, probably because the α-amino group and residues D4 and/or D5 have been replaced (cf. Fig. 3).

Peptides that bind mAb M5 but do not bind mAbs M1 and M2 include MDFKDDDDK and MDYRDDDDK (Table 3, nos. 11 and 12). Using a set of peptides in which individual residues were replaced by a structurally related one, i.e. D by E, Y by F and K by R, it was found that peptides containing F2 and/or R3 only bind mAb M5. Since Y2 and K3 are critical for binding mAbs M1 and M2, peptides containing F2 and/or R3 are not recognized by these mAbs (cf. Fig. 3).

Peptides that bind mAbs M2 and M5 but do not bind mAb M1 include MDYKDDDDK and MDYKDDDDA (Table 3, nos. 1 and 6). These peptides do not bind mAb M1, probably because the α-amino group has been replaced (cf. Fig. 3). Various other peptides that contain the consensus sequence MDYKDDDXX also only bind mAbs M2 and M5 (Table 3).

Peptides that bind mAbs M1 and M2 but do not bind mAb M5 include DYKAFDNL and DYKHLDNS (Table 2, nos. 20–23). These peptides do not bind mAb M5, probably because the residues D4 and/or D5 have been replaced (cf. Fig. 3).

Peptides that bind mAbs M1 and M5 but do not bind mAb M2 were not found. Combining the important residues described above, it seems difficult to design such peptides.

Peptides that bind mAbs M1, M2 and M5 include the original peptide DYKDDDDK and some of the peptides in which individual amino acids were replaced by structurally related amino acids (Table 1, nos. 1 and 13–17). These peptides contain most of the critical residues for all

three mAbs. Another peptide that binds mAbs M1 and M2 and, to a lesser extent, mAb M5, was MDYKDKDDK (Table 3, no. 8). This peptide has the advantage that it starts with the translational start residue methionine.

The sensitivity in ELISA of mAb M1 for MDYKDKDDK was increased compared to that of MDYKDDDDK (Table 3, cf. no. 1 with no. 8). This is consistent with recent data showing that, compared to DYKDDDDK, the peptide DYKDKD has a significantly increased affinity for mAb M1 [9].

Two examples of a putative application of the new FLAG peptide variants are shown in Fig. 4. The examples include simultaneous and separate detection of two co-expressed proteins (Fig. 4A) and separate detection of the precursor form and the mature form of a particular protein (Fig. 4B).

Improved tag sequences

Previously, it has been shown that the sensitivity in ELISA for mAb M1 can be improved significantly using variants of the FLAG peptide. For example, the sensitivity in Western blots of DYKDE attached to the amino terminus of mAbs was increased sixfold over the original FLAG sequence [8]. Furthermore, in competitive ELISAs many new peptides were identified that showed a 10- to 100-fold increased activity [9]. Our study revealed additional new peptides for which the sensitivity in direct ELISA for mAbs M2 and M5 was increased 10-fold. With mAb M2, the sensitivity in ELISA of peptides including MDYKAFDNL was increased 10-fold over that of MDYKDDDDK (Fig. 1A). With mAb M5, the sensitivity in ELISA of peptides including MDFKDDDDK and MDYRDDDDK was increased 10-fold (Fig. 1B).

To obtain more insight in the binding affinity of some of the above described new FLAG peptide variants, competitive ELISAs were performed with three peptides recognized by anti-FLAG M2. The first two peptides, DYKDDDDK and MDYKDDDDK, are the original two FLAG peptides. The third peptide, MDYKAFDNL, was identified using the semi-random library. This third peptide showed a 10-fold improved binding activity in three different types of direct ELISAs (pin ELISA, microtiter ELISA and the dotting immuno assay; cf. Figs. 1 and 2). Surprisingly, it was found that MDYKAFDNL as a soluble peptide has a much lower binding activity. In solution the peptides DYKDDDDK and MDYKDDDDK had IC_{50}'s that were 10-fold better than that of MDYKAFDNL (Table 5). Recently it has been shown that a relatively 'unstructured' conformation of synthetic peptides can be stabilized by immobilization of the peptide on a carrier [20,21]. This would explain the increased recognition of mAb M2 and MDYKAFDNL when synthesized or coated onto a solid phase. It would also explain the high IC_{50}'s obtained when MDYKAFDNL is coated (Table 5). A high binding affinity between mAb M2 and solid-phase MDYKAFDNL would require much more soluble peptide to obtain 50% inhibition of binding.

An important question is whether MDYKAFDNL or any of the other identified new variants can be used successfully as affinity tags. We think this to be the case, because affinity tags are fused to a carrier. This resembles the way in which the peptide is presented to the mAb in the pin ELISA, i.e. the carboxy terminus of the peptide is covalently linked whereas the rest of the peptide is 'free' in solution. However, actual usage of these peptides in expression systems will show whether this is indeed the case.

Conclusions

Using the pin ELISA binding of mAbs M1, M2 and M5 to variants of the FLAG peptide epitope, tags were investigated. Since the selectivities in ELISA are in good agreement with the binding activity found using various other techniques, we postulate that the selective and differential recognition properties of the new tag sequences reported here may be used to separately detect, quantify and purify co-expressed recombinant proteins. In addition, our studies show that the way in which a peptide is presented to an antibody, e.g. linked to a pin, coupled to a microtiter plate or to nitrocellulose paper, or as soluble peptides, may strongly contribute to the binding activity observed between peptides and their antibodies.

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