J. Biochem. 2009;145(5)693–700
doi:10.1093/jb/mvp027

Epitope Mapping Using Ribosome Display in a Reconstituted Cell-Free Protein Synthesis System

Eriko Osada, Yoshihiro Shimizu*, Bintang K. Akbar, Takashi Kanamori and Takuya Ueda

The Department of Medical Genome Sciences, Graduate School of Frontier Sciences, the University of Tokyo, FSB-401, 5-1-5, Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

Received January 21, 2009; accepted February 6, 2009; published online February 19, 2009

Ribosome display is a powerful technology for selecting ligand-binding peptides or proteins. We demonstrate here that the ribosome display using the reconstituted cell-free protein synthesis system can be applied for the epitope mapping of monoclonal antibodies (mAbs). Using this technology, we selected peptides that specifically bind to three mAbs from random peptide library. When selection was performed against the anti-FLAG M2 antibody, selected peptides contained previously characterized consensus epitope, indicating that the methodology can be applied for the epitope mapping. When the selection was carried out against two anti-β-Catenin (anti-β-Cat) mAbs, selected peptides had a homology for the partial peptide sequences of β-Cat. Western blot analysis showed that these putative epitopes had affinity for the corresponding mAbs and β-Cat mutants that lack these regions did not bind to the antibodies, indicating we correctly mapped the epitope for these mAbs. The study shown here provides a way for the quick identification of the epitope of mAbs.

Key words: antibody, cell-free protein synthesis system, epitope mapping, in vitro selection, ribosome display.

Abbreviations: β-Cat, β-Catenin; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; pD, protein D; PURE system, protein synthesis using recombinant elements system; SD, Shine-Dalgarno; HRP, horseradish peroxidase; PBST, Phosphate Buffered Saline with Tween 2D.

Antibodies are the macromolecules that can bind with high affinity and specificity to diverse ligand molecules. In line with the development of molecular biology and biotechnology, they have become a powerful tool for basic researches, diagnosis, pharmaceutics and treatment of intractable diseases (*1*, *2*). The interaction between antibodies and antigens is based on the rearrangement of the sequence of variable regions of the antibodies. These regions mainly interact with the specific surface of the antigen that is known as epitopes.

Identification of the epitope, a process called as epitope mapping, is a key step in characterization of the monoclonal antibodies (mAbs) (*2*). It determines key amino acid residues in the antigen that are important for antibody recognition and binding. The information derived from this method is essential for the understanding of the molecular basis of antibody–antigen interactions and the development of effective synthetic peptide vaccines.

There are a number of ways for identifying epitopes against mAbs. Determination of the 3D structure of the complex comprised of antibody and antigen using X-ray crystallography or NMR analysis is effective for completely understanding the molecular basis of the interaction. The method using synthetic peptide arrays is also applied to the epitope mapping (*3*). However, these approaches require the preparation of high homogenous samples for the structural analysis or diverse peptide candidates for the mapping. Therefore, these approaches are not suitable for rapid identification of the diverse mAbs. Phage display is another way for the epitope identification (*4–6*). The method is based on a physical linkage between phenotype and genotype in a phage particle. Using affinity selection of peptides displayed on a phage surface, sequences of peptides that are able to bind with the mAbs can be derived by sequencing the cDNA clones. Since the method allows the selection from a large library that encodes diverse peptides ($\sim 10^8$), it is utilized for a number of epitope identification.

In the past decade, *in vitro* display technologies using a cell-free protein synthesis system, such as ribosome display (*7*), *in vitro* virus (*8*) or mRNA display (*9*), DNA display (*10*) and CIS display (*11*) have been developed for the peptide selection. The basis of the selection strategy of these methodologies is same as the phage display, a physical linkage between genotype and phenotype, although the format of the linkage is unique in each method. Nevertheless, cell-free methods stand as an attractive tool in that all screening processes are performed *in vitro*. Many of the limitations with regard to those using living cells, such as the library size is limited due to low transformation efficiency, the toxic protein cannot be expressed, deflection of the library takes place during the growth rate of the living cells, can be circumvented.

*To whom correspondence should be addressed. Tel: +81-4-7136-3649, Fax: +81-4-7136-3648, E-mail: shimizu@k.u-tokyo.ac.jp

In the present study, we demonstrate that a PURE (protein synthesis using recombinant elements) ribosome display approach (*12–14*) can be utilized for the epitope mapping of mAbs whose epitope is both previously identified and unidentified. The PURE system, a reconstituted cell-free protein synthesis system composed of essential factors and enzymes for gene expression in *Escherichia coli* (*15*, *16*), has been proposed to be a best format for the *in vitro* display technologies due to its high controllability and reduced nuclease activities. Using this system, a rapid, simple and precise procedure for the epitope identification of several mAbs is presented.

MATERIALS AND METHODS

Construction of Random Peptide Library—A gene for the spacer sequence [protein D (pD), a phage Lambda capsid protein] (*17*), kindly provided by Dr Matsuura (Osaka University), and the subsequent SecM arrest sequence was sub-cloned into pURE1 vector (post-genome institute) between BamHI and XhoI site. The resultant plasmid was used for the amplification of the library that encodes 10 amino acid random peptide sequences followed by the spacer sequence. Using 10_aa_library primer (5′-GAAGGAGATATACCATATG - $(NNK)_{10}$ - GGATCCGGTGGCGGTTCCG-3′; N is any nucleotide and K is either G or T, respectively) and SecM_25_RD_RT3 primer (5′-AAGCTTCTCGAGCCCGG TGAGGCGT-3′), a library sequence was amplified and purified by gel electrophoresis. The products were used as templates for the second step PCR. Using Universal primer (5′-GAAATTAATACGACTCACTATAGGGAGACC ACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT AAGAAGGAGATATACCA-3′) and SecM_25_RD_RT3 primer, a library was amplified and purified by gel electrophoresis. The products were used as templates for the ribosome display selection in the PURE system.

Sample Preparation—The components used in the PURE system were purified as previously described (*15*). Ribosome was prepared according to the previous report (*12*). Biotinylated anti-FLAG M2 antibody and the FLAG peptide were purchased from Sigma, St Louis, Mo, USA. β-Catenin (β-Cat) or its deletion mutants were prepared as follows: The plasmid encoding a gene for β-Cat with a His-tag at its N-terminus was kindly provided by Dr Masuho (Tokyo University of Science). Deletion mutants were obtained by overlap extension PCR according to Heckman *et al.* (*18*). Obtained plasmids were transformed into *E. coli* strain Rosetta(DE3)/pLysS (Stratagene, La Jolla, CA, USA). The protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were grown for 15 h at 18°C in LB broth. Overexpressed β-Cat was purified with Ni-NTA agarose (Qiagen, Hilden, Germany), as recommended by the manufacturers, and further purified by anion-exchange chromatography using mono-Q column (GE-Healthcare, Little Chalfont, UK) with linear gradient elution from buffer A (20 mM sodium phosphate, pH 7.4, 100 mM KCl, 7 mM 2-mercaptoethanol) to buffer B (20 mM sodium phosphate, pH 7.4, 460 mM KCl, 7 mM 2-mercaptoethanol). Eluted protein was dialyzed against PBS buffer with 20% glycerol and stored at –80°C for later use. Preparation of mouse anti-β-Cat monoclonal antibodies was as follows: hybridoma cells secreting specific antibodies, kindly provided by Dr Masuho (Tokyo University of Sciences), were cultured in GIT medium (Nihon Pharmaceutical Co., Ltd, Tokyo, Japan). Immunoglobulin isotypes were determined by IsoStrip (Roche, Penzberg, Germany). The mAbs were purified by affinity chromatography using Protein G-Sepharose (GE-Healthcare, Little Chalfont, UK) from hybridoma supernatants.

Ribosome Display Selection—Amplified PCR products were translated in 20 μl transcription/translation coupled the PURE system for 20 min at 37°C. The reaction condition of the PURE system was as described previously (*16*). The reaction was stopped by the addition of 100 μl of ice-cold Stop buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $Mg(OAc)_2$, 0.1% Tween-20, 2.5 mg/ml heparin sodium]. The reaction mixtures were then centrifuged at 14,000*g* for 10 min to remove insoluble components. To eliminate non-specific ternary complexes, the supernatant was then incubated with 50 μl magnetic beads without ligands for 30 min at room temperature. Remained supernatant was incubated with monoclonal antibody-immobilized magnetic beads for 1 h at room temperature. Dynabeads M-280 Streptavidin (Invitrogen, Carlsbad, CA, USA) was used for the immobilization of biotinylated anti-FLAG M2 antibody and Dynabeads Protein G (Invitrogen, Carlsbad, CA, USA) was used for the immobilization of anti-β-Cat mAbs. The beads were then washed five times with Wash buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 50 mM $Mg(OAc)_2$]. Elution of the ternary complex was carried out by the addition of Wash buffer with 100 μg/ml FLAG peptide or 20 nM β-Cat in the case of competitive elution. After DNA was completely digested with DNase I (Ambion, Austin, TX, USA) for 30 min at 37°C, mRNA was recovered using RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany). RT-PCR was performed by SuperScript III Reverse Transcriptase (Invitrogen, Hilden, Germany) using adequate primers. The amplified product was purified by gel electrophoresis and used for the next round of selection. Cloning of the selected library was performed by TOPO TA cloning kit for Sequencing (Invitrogen, Hilden, Germany).

ELISA and Western Analyses—The DNA that encodes selected peptide followed by the spacer sequence and subsequent V5 epitope tag was amplified from each clone using universal primer and pD_V5 primer (5′-CTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGAT AGGCTTACCAACGATGCTGATTGCCGTTCC-3′). The amplified DNA was then translated in the PURE system and the synthesized peptides were used for ELISA (enzyme-linked immunosorbent assay) analysis or western analysis. ELISA analysis was performed as follows: Nunc Immobilizer Streptavidin plate (Thermo Fisher Scientific Inc., Waltham, MA, USA) was incubated with biotinylated anti-FLAG M2 antibody in PBS for 30 min at room temperature. The plate was then blocked with PBS containing 1% BSA (Sigma, St Louis, MO, USA) and washed with PBST. The synthesized peptides were then added to the plate and incubated for 2 h at

room temperature. The plate was washed with PBST and bound peptides were detected with 1-Step Turbo TMB ELISA kit (Pierce, Rockford, IL) using HRP-conjugated anti-V5 antibody (Invitrogen, Hilden, Germany). Western blot analysis was performed as follows: The V5-tagged synthesized peptides were separated by 15% SDS–PAGE and then electrotransferred to a nitrocellulose membrane (Tokyo Roshi, Tokyo, Japan). The membrane was blocked with Block Ace (Nihon Pharmaceutical Co., Ltd, Tokyo, Japan) and washed with PBST. The synthesized peptides were detected with anti-β-Cat mAbs or HRP-conjugated anti-V5 antibody. In the case of the detection by anti-β-Cat mAbs, HRP-conjugated goat anti-mouse IgG antibody (Biosource, San Jose, CA, USA) was used as a secondary antibody. The immunoblots were developed using the Immobilon western chemiluminescent HRP substrate kit (Millipore, Billerica, MA, USA) according to the instructions of the supplier.

RESULTS

Ribosome Display Selection Against the Anti-FLAG M2 Antibody—As a control experiment, we performed a ribosome display selection of the peptide library against the anti-FLAG M2 antibody that is known to have preference for the DYKXXD sequence where X is an arbitrary amino acid (*19*). As shown in Fig. 1A, a library of 10^{11} DNA molecules, which encodes 10 amino acids random peptide sequences followed by a spacer sequence [pD, a phage Lambda capsid protein, (*17*)], was used as the initial input in a first round selection. The selection was based on a ternary complex formation composed of mRNA, ribosome and peptidyl-tRNA formed by the SecM elongation arrest sequence [amino acid residues 148–170, (*20*)] that was inserted downstream from the spacer sequence (Fig. 1A). The ternary complex formation was performed by the PURE system, a reconstituted cell-free protein synthesis system that is suitable for the *in vitro* selection technologies due to its reduced nuclease activities (*12–16*). These complexes were subjected to an affinity purification using biotinylated anti-FLAG M2 antibody immobilized on the streptavidin-coated magnetic beads as a target molecule and the FLAG peptide as an eluent. Recovered mRNAs were then amplified by RT-PCR and used for the next round of the selection.

Three rounds of selection were performed. After each round of selection, the selected library was V5-tagged (Fig. 1A) and translated in the PURE system to analyse the enrichment efficiency by ELISA. As shown in Fig. 1B, the fraction of the library that bound with immobilized anti-FLAG M2 antibody in ELISA was increased after each selection round. Drastic enrichment was observed after the second round of selection. After the third round of selection, the absorbance in ELISA reached 80% of the FLAG epitope sequence (DYKD DDDK). These results suggest that the specific enrichment of the library was achieved during the selection rounds.

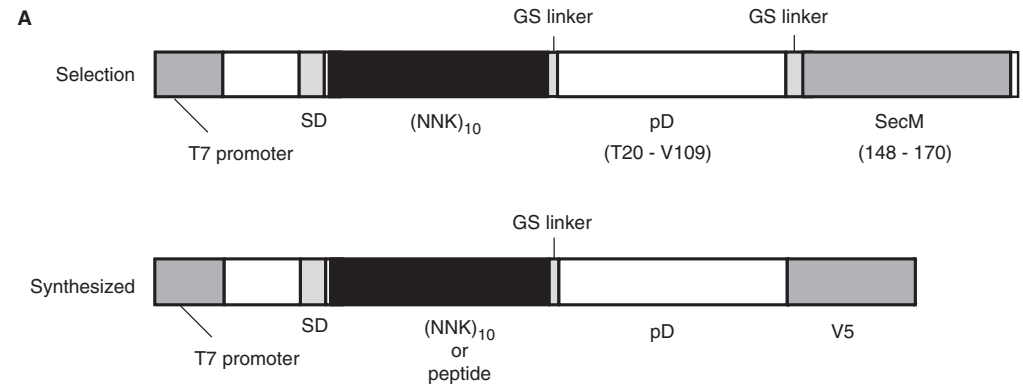
The library after the second round of selection was cloned and sequenced (Fig. 1C). Out of 19 randomly picked clones, 12 clones possessed the consensus epitope sequence for the anti-FLAG M2 antibody (DYKXXD), six possessed a partial epitope sequence and only one did not possess any consensus sequence. This result indicates that our approach can be adopted for the epitope mapping with other antibodies whose epitope has not been identified.

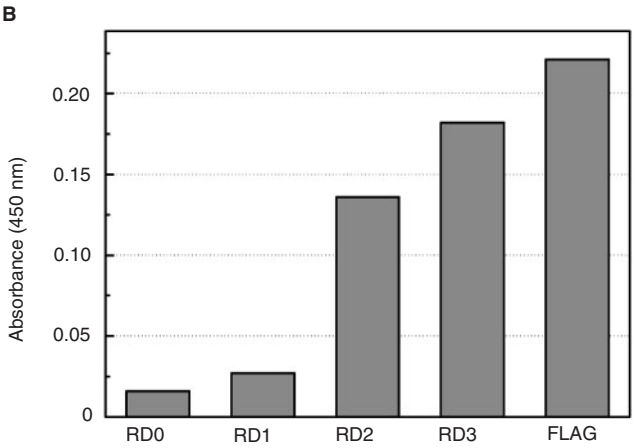
Ribosome Display Selection against Anti-β-Cat mAbs—To demonstrate that the ribosome display selection of the random peptide library using the PURE system is capable of determining the epitope sequence against antibodies whose epitope is unidentified, we chose uncharacterized anti-β-Cat mAbs (the mAb 36a and the mAb 48a) as an antibody target for selection. β-Cat is an oncogenic protein that plays an important role in the Wnt signalling pathway and is an important component of the cadheline cell-adhesion complex (*21*).

A library of 10^{11} DNA molecules that encodes 10 amino acid random peptide sequences were used as the initial input in a first round of selection, and three rounds of selection were carried out. Selection was performed by affinity purification of the ternary complexes comprised of mRNA, ribosome and peptidyl-tRNA. Antibodies, mAb 36a or mAb 48a, that were immobilized on the Protein G-coated magnetic beads and the solution containing purified β-Cat were used for washing out the ternary complex. After each rounds of selection, the selected library was V5-tagged in the process of PCR amplification and translated to the PURE system to analyse the enrichment efficiency by western blot analysis. As shown in Fig. 2A and B, round one selection output showed reactivity towards the antibodies used for the affinity selection in both cases, suggesting that specific enrichment of the library was achieved in an early round of selection.

Sequence Analyses of Selected Peptides—The libraries after the third round of selection against the mAb 36a or the mAb 48a were cloned and sequenced. In both cases, the libraries were highly enriched and converged to restricted sequences. Three sequences were derived in the case of the mAb 36a and two sequences were found in the case of the mAb 48a (Fig. 2C and D). Although we have no obvious explanation for high convergence of the selected sequences from the library of 10^{11} molecules, one possible reason may be the selection step by the addition of the ligand-free magnetic beads (see MATERIALS AND METHODS section), which has not been performed in the previous report (*12*) and might result in lower recovery rate of the ribosome complex. We sequenced the libraries after the first round of selection. In this case, the libraries contained diverse sequences involving unspecific sequences that may not bind to the target antibodies (data not shown).

Irrespective of the selection round, obtained sequences contained putative epitope motifs that were found in the sequence of the original antigen, β-Cat. In the case of the mAb 36a, STKFD sequence, which has a homology for the $\beta\text{-Cat}^{111–115}$ (STQFD; amino acid residues 111–115 of β-Cat), was observed in the selected peptides (Fig. 2C). The YLDS sequence that is identical to the $\beta\text{-Cat}^{29–32}$ (YLDS; amino acid residues 29–32 of β-Cat) was also found (Fig. 2C). In the case of the mAb 48a, TLVMDL NAIE sequence and a related sequence (TLVVGLDAID),





C

				Epitope Motif									
				D	**Y**	**K**	**X**	**X**	**D**				
				D	**Y**	**K**	G	L	**D**	R	G	V	I
				D	**Y**	**K**	F	E	**D**	N	L	E	C
				D	**Y**	**K**	F	L	**D**	E	V	Q	N
				D	**Y**	**K**	W	E	**D**	K	V	C	I
A	R	I	L	**D**	C	**K**	M	D	**D**	D			
				D	**Y**	**K**	M	M	**D**	R	R	D	F
				D	**Y**	**K**	C	H	**D**	T	G	T	D
A	L	R	S	**D**	**Y**	**K**	W	T	**D**				
			V	**D**	**Y**	Q	L	R	G	A	G	Y	
				D	**Y**	**K**	S	T	**D**	K	W	G	Y
				D	**Y**	**K**	P	S	V	N	E	G	G
A	Y	A	I	**D**	**Y**	**K**	R	T	**D**				
	I	S	R	**D**	**Y**	**K**	V	D	**D**	A			
			G	**D**	**Y**	**K**	F	A	**D**	D	M	A	
T	S	H	W	**D**	**Y**	**K**	C	W	E				
				S	**Y**	**K**	D	F	**D**	L	R	K	G
				D	**Y**	**K**	F	M	G	Y	V	A	S
		F	D	**D**	**Y**	**K**	M	T	**D**	I	Y		

Fig. 1. **Ribosome display selection against the anti-FLAG M2 antibody.** (A) DNA constructs used for the ribosome display selection and characterization of the selected peptide sequences. SD represents Shine–Dalgarno sequence, pD represents the phage Lambda capsid protein D and V5 represents the V5 epitope tag. (B) ELISA of selected peptides after each round of ribosome display selection. Translation products of an initial library and rounds 1, 2 and 3 selection output were analysed. The FLAG peptide fused to the spacer and V5 epitope tag was also analysed as a positive control. (C) Sequence analysis of the selected peptides. Isolated clones after the second round of selection were sequenced. Sequences with FLAG epitope motif are boxed.

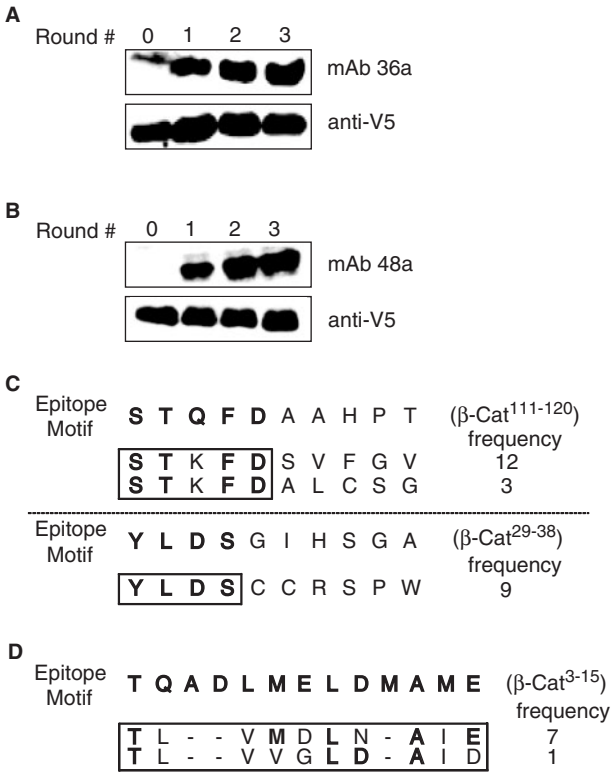


Fig. 2. **Ribosome display selection against β-Cat mAbs.** (A, B) Western blot analysis of the selected peptides. Translation products of an initial library and rounds 1, 2 and 3 selection output were analysed by using anti-V5 antibody. Ribosome display selection was performed against the mAb 36a (A) and the mAb 48a (B). (C, D) Sequence analysis of the selected peptides. Isolated clones after the third round of selection against the mAb 36a (C) and the mAb 48a (D) were sequenced. Selected peptides, as well as the putative epitope motifs encoded in the original antigen are shown. Partial regions similar to the putative epitope motif are boxed. Frequency with which each clone was identified is also shown.

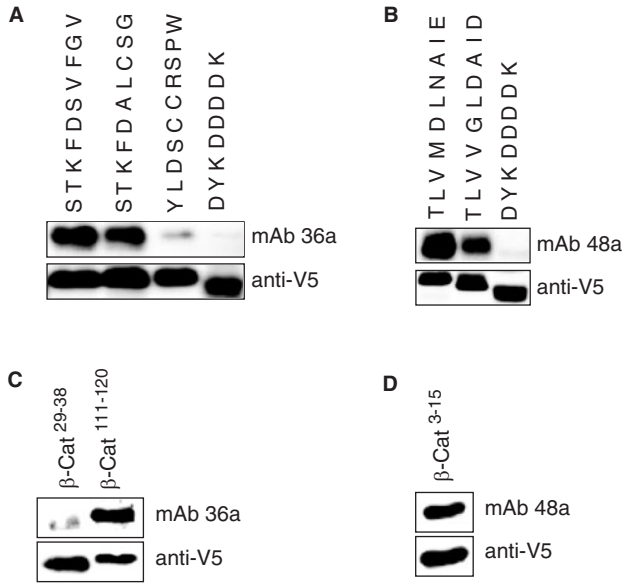


Fig. 3. **Characterization of the selected peptides.** The selected peptides (A, B) and the putative epitope motifs fused to the spacer and the V5 epitope tag (C, D) were analysed by western blot analysis using the mAb 36a (A, C) and the mAb 48a (B, D). Total quantity of synthesized peptide was also analysed by using the anti-V5 antibody. DYKDDDDK indicates the result of FLAG peptide fused to the spacer and the V5 epitope tag as a negative control.

which have a homology for the β-Cat$^{3–15}$ (TQADLMEL DMAME; amino acid residues 3–15 of β-Cat), were found to be enriched (Fig. 2D).

Characterization of Selected Peptides and Epitope Mapping—To address whether the selected peptides are accurately recognized by the corresponding antibodies, they were synthesized in the PURE system as a V5-tagged form (Fig. 1A) and analysed by western analysis (Fig. 3). The results indicated that the peptides possessing the STKFD sequence (STKFDSVFGV and STKFD ALCSG) were able to bind with the mAb 36a (Fig. 3A). Although the affinity was lower than those with the STKFD sequence, the peptide possessing the YLDS sequence (YLDSCCRSPW) also interacted with the mAb 36a (Fig. 3A). In the case of the peptides with homology for β-Cat$^{3–15}$ (TLVMDLNAIE and TLVVGLDAID), they interacted with the mAb 48a (Fig. 3B).

According to above results, we synthesized the V5-tagged partial peptides β-Cat$^{111–120}$ (STQFDAAHPT; amino acid residues 111–120 of β-Cat), β-Cat$^{29–38}$ (YLD SGIHSGA; amino acid residues 29–38 of β-Cat) and β-Cat$^{3–15}$ (TQADLMELDMAME; amino acid residues 3–15 of β-Cat) in the PURE system and analysed their interaction with the mAb 36a or the mAb 48a by western blot analysis (Fig. 3C and D). The results showed that the β-Cat$^{111–120}$ and the β-Cat$^{3–15}$ specifically interacted with the mAb 36a and the mAb 48a, respectively. Although the results also indicated that β-Cat$^{29–38}$ shows reactivity towards the mAb 36a, the interaction was considerably weaker than that of the β-Cat$^{110–120}$. These data suggest that the β-Cat$^{111–120}$ contains an epitope for the mAb 36a and the β-Cat$^{3–15}$ contains an epitope for the mAb 48a.

We further prepared β-Cat variants that lack the suggested region and they were tested for the binding of mAbs. The results (Fig. 4A and C) showed that β-Cat$^{\Delta111–115}$ that lacks amino acid residues 111–115 of β-Cat did not bind to the mAb 36a, and β-Cat$^{\Delta3–15}$ that lacks amino acid residues 3–15 did not bind to the mAb 48a. From these results, we conclude that the epitope for the mAb 36a is the region that includes amino acid residues 111–115 and the epitope for the mAb 48a is the one that includes amino acid residues 3–15 of β-Cat.

To precisely map the epitope for the mAb 36a and the mAb 48a, a series of deletion mutants of β-Cat were prepared and tested for the binding of the mAb 36a or the mAb 48a. As shown in Fig. 4B, β-Cat$^{\Delta111}$ and β-Cat$^{\Delta115–120}$ did not bind to the mAb 36a, while β-Cat$^{\Delta116–120}$ did, indicating that Ser111 and Asp115 are essential for the binding. In the case of the mAb 48a (Fig. 4D), β-Cat$^{\Delta3–6}$ and β-Cat$^{\Delta14–15}$ did not bind to the

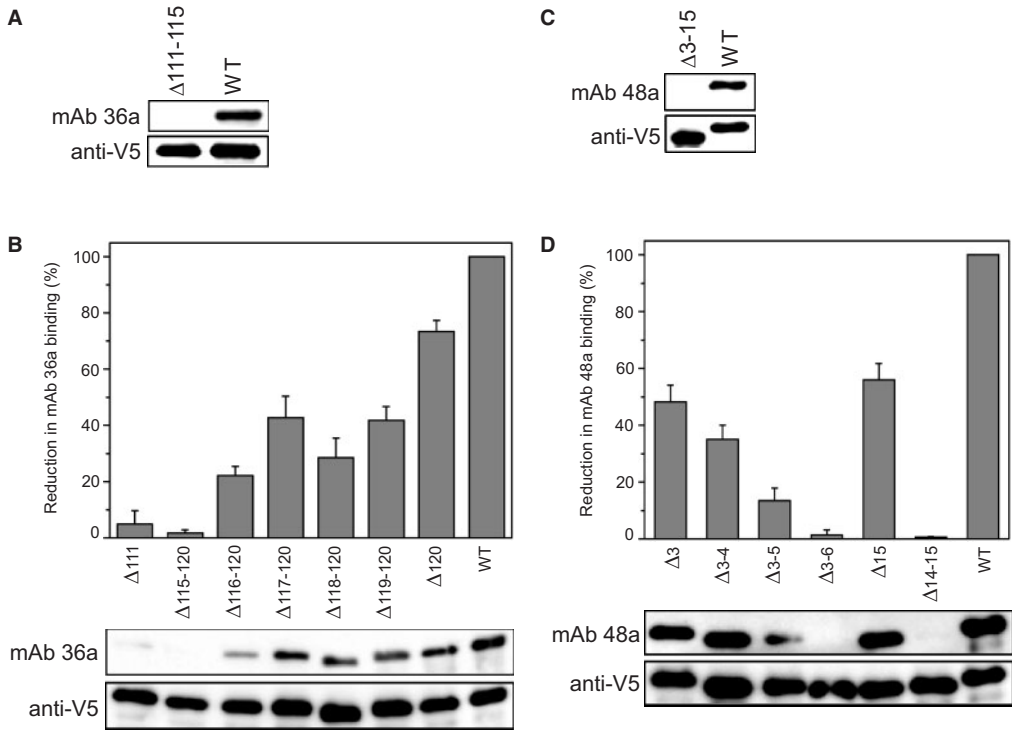


Fig. 4. **Western blot analysis of the β-Cat mutants that lack the putative epitope motifs.** Each mutants were analysed by the mAb 36a (A, B) and the mAb 48a (C, D). Total quantity of expressed mutants was also analysed by using the anti-V5 antibody.

mAb 48a whereas β-Cat$^{\Delta 3-5}$ showed weak reactivity and β-Cat$^{\Delta 3-4}$ and β-Cat$^{\Delta 15}$ specifically bound to the mAb 48a, indicating that Asp6 and Met14 are crucial for the binding. These results demonstrated that β-Cat$^{111-115}$ forms a core epitope for the mAb 36a, and β-Cat$^{6-14}$ forms that for the mAb 48a.

DISCUSSION

We have described here that the ribosome display using the reconstituted cell-free protein synthesis system can be applied for the epitope mapping of mAbs. Using random peptide libraries, several mimotopes have been selected against the anti-FLAG M2 antibody and anti-β-Cat mAbs.

In the case of the anti-FLAG M2 antibody, about 60% of the selected clones contained the consensus FLAG epitope motif (DYKXXD) suggesting specific enrichment is achieved only in the two selection rounds (Fig. 1B and C). Since the ratio of the sequence involving such motif in the initial pool can be estimated to be $\sim 10^{-5}$, the result is in accordance with the high enrichment efficiency (10^3- to 10^4-fold per round) of the ribosome display selection using the PURE system that was reported previously (*12*). In addition, we have succeeded to obtain specific epitope sequences against anti-β-Cat mAbs in three selection rounds (Figs 2 and 3). Unfortunately, the mapped regions against two anti-β-Cat mAbs (β-Cat$^{3-15}$ and β-Cat$^{111-120}$) are not on a structural core of armadillo repeats (*22*), which is crucial for the interaction with diverse proteins involved in cell adhesion and Wnt signalling pathway. This indicates that the antibodies used here may not affect the function of the β-Cat. However, we consider that the present results suggest that our approach provides a way for the quick identification of the epitope against sequence-specific mAbs.

By using a series of deletion mutants of β-Cat, we have mapped the epitopes for two mAbs. According to the results (Fig. 4B and D), β-Cat$^{111-115}$ (STQFD) forms a core epitope for the mAb 36a, and β-Cat$^{6-14}$ (DLMELDMAM) forms that for the mAb 48a. Since the selected peptides against the mAb 36a contain STKFD sequence, the result of the mAb 36a suggests that STXFD (X is an arbitrary amino acid) sequence might be an epitope for this monoclonal antibody. On the other hand, the selected peptides against the mAb 48a do not contain the amino acids corresponding to Asp6 and Met14 that are suggested to be crucial for the interaction with the antibody. To clarify the reason of this inconsistency, we investigated the significance of each amino acid residue by an alanine scanning mutagenesis experiment (Supplementary Fig. 1). The data shows that Asp6, Leu10, Asp11 and Met14 of β-Cat$^{3-15}$ are crucial amino acids (Supplementary Fig. 1A) whereas Thr1, Asp5, Leu6, Ile9 and Glu10 of one of the selected peptide (TLVMDLNAIE) are essential (Supplementary Fig. 1B). As shown in Supplementary Fig. 1C, it is possible that Asp6, Leu10 and Asp11 of β-Cat$^{3-15}$ and Asp5, Ile9 and Glu10 of the selected peptide support the binding in a same manner, respectively. The lack of the amino acid

corresponding to Met14 of β-Cat$^{3-15}$ might be complemented by Thr1 and Leu6 of the selected peptide, nevertheless, further analyses are necessary to elucidate the binding mechanism of these peptides with the antibody.

Practically, the epitope mapping by using ribosome display selection has diverse advantages over other conventional approaches. The selection of the peptide and the amplification of the genes encoding selected peptide can be achieved in 1 day, which takes at least 2 days when it is performed by phage display. Moreover, the initial library is a PCR-amplified product, which is synthesized with random DNA primers. Therefore, it can be prepared at low cost compared with the method using synthetic peptides and its size can be highly increased than any other conventional methods.

The use of the reconstituted cell-free protein synthesis system has an additional advantage in the characterization step of the selected epitope. Since false positive binders are often selected in the *in vitro* display selection, this step is essential for the verification of the selection. In the present study, the selected peptides have been synthesized directly from the isolated clones by using the PURE system, subjected directly to SDS–PAGE, and analysed by western blot analysis (Figs 2 and 3). Such an approach does not require the overexpression and purification of the peptide, or the chemical synthesis step that might be laborious and time consuming. In particular, a number of peptides can be investigated simultaneously simply by adding PCR-amplified DNA into the PURE system. Furthermore, in the PURE system, such DNA does not require the additional spacer sequences to protect from degradation by any deoxynucleases. We consider that these features, as well as a simple strategy to form highly stable ternary complex composed of ribosome, mRNA and peptidyl-tRNA in the ribosome display selection (*12*), may lead to the construction of high-throughput epitope mapping system at low cost in the future. It is also noteworthy that the PURE system does not contain any proteases so that the selected peptides are protected from degradation.

In summary, the methodology shown here might be readily compatible for the practical epitope mapping of diverse mAbs. All the steps can be carried out *in vitro* and therefore, the scientist can obtain the epitope information rapidly by using this technology. We believe that the presented results will have a significant impact on applications dealing with antibodies and support the development of the research field of antibody engineering.

SUPPLEMENTARY DATA

Supplementary Data are available at *JB* Online.

ACKNOWLEDGEMENTS

We thank Yasuhiko Masuho and Yukari Kouya (Tokyo University of Science) for providing β-Cat expression plasmid and hybridoma cell lines, 36a and 48a and Tomoaki Matsuura (Osaka University) for providing a plasmid encoding lambda phage pD sequence.

FUNDING

City Area Program from the Ministry of Education, Science, Sports and Culture of Japan.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Brekke, O. and Loset, H.G.A. (2003) New technologies in therapeutic antibody development. *Curr. Opin. Pharmacol.* **3**, 544–550
2. Nelson, P.N., Reynolds, G.M., Waldron, E.E., Ward, E., Giannopoulos, K., and Murray, P.G. (2000) Monoclonal antibodies. *Mol. Pathol.* **53**, 111–117
3. Hilpert, K., Winkler, D.F., and Hancock, R.E. (2007) Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nat. Protoc.* **2**, 1333–1349
4. Rowley, M.J., O'Connor, K., and Wijeyewickrema, L. (2004) Phage display for epitope determination: a paradigm for identifying receptor-ligand interactions. *Biotechnol. Annu. Rev.* **10**, 151–188
5. Mullen, L.M., Nair, S. P., Ward, J.M., Rycroft, A.N., and Henderson, B. (2006) Phage display in the study of infectious diseases. *Trends Microbiol.* **14**, 141–147
6. Sidhu, S.S., Fairbrother, W.J., and Deshayes, K. (2003) Exploring protein-protein interactions with phage display. *Chembiochem.* **4**, 14–25
7. Hanes, J. and Plückthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl Acad. Sci. USA* **94**, 4937–4942
8. Nemoto, N., Miyamoto-Sato, E., Husimi, Y., and Yanagawa, H. (1997) In vitro virus: bonding of mRNA bearing puromycin at the 3'-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro. *FEBS Lett.* **414**, 405–408
9. Baggio, R., Burgstaller, P., Hale, S.P., Putney, A.R., Lane, M., Lipovsek, D., Wright, M.C., Roberts, R.W., Liu, R., Szostak, J.W., and Wagner, R.W. (2002) Identification of epitope-like consensus motifs using mRNA display. *J. Mol. Recognit.* **15**, 126–134
10. Yonezawa, M., Doi, N., Kawahashi, Y., Higashinakagawa, T., and Yanagawa, H. (2003) DNA display for in vitro selection of diverse peptide libraries. *Nucleic Acids Res.* **31**, e118
11. Odegrip, R., Coomber, D., Eldridge, B., Hederer, R., Kuhlman, P.A., Ullman, C., FitzGerald, K., and McGregor, D. (2004) CIS display: in vitro selection of peptides from libraries of protein-DNA complexes. *Proc. Natl Acad. Sci. USA* **101**, 2806–2810
12. Ohashi, H., Shimizu, Y., Ying, B.W., and Ueda, T. (2007) Efficient protein selection based on ribosome display system with purified components. *Biochem. Biophys. Res. Commun.* **352**, 270–276
13. Matsuura, T., Yanagida, H., Ushioda, J., Urabe, I., and Yomo, T. (2007) Nascent chain, mRNA, and ribosome complexes generated by a pure translation system. *Biochem. Biophys. Res. Commun.* **352**, 372–377
14. Villemagne, D., Jackson, R., and Douthwaite, J. A. (2006) Highly efficient ribosome display selection by use of purified

components for in vitro translation. *J. Immunol. Methods.* **313**, 140–148
15. Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* **19**, 751–755
16. Shimizu, Y., Kanamori, T., and Ueda, T. (2005) Protein synthesis by pure translation systems. *Methods.* **36**, 299–304
17. Forrer, P. and Jaussi, R. (1998) High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D. *Gene.* **224**, 45–52
18. Heckman, K.L. and Pease, L.R. (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat. Protoc.* **4**, 924–932
19. Slootstra, J.W., Kuperus, D., Plückthun, A., and Meloen, R.H. (1997) Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5. *Mol. Divers.* **2**, 156–164
20. Nakatogawa, H. and Ito, K. (2002) The ribosomal exit tunnel functions as a discriminating gate. *Cell* **108**, 629–636
21. Megy, S., Bertho, G., Gharbi-Benarous, J., Baleux, F., Benarous, R., and Girault, J.P. (2006) STD and TRNOESY NMR studies for the epitope mapping of the phosphorylation motif of the oncogenic protein beta-catenin recognized by a selective monoclonal antibody. *FEBS Lett.* **580**, 5411–5422
22. Xing, Y., Takemaru, K., Liu, J., Berndt, J.D., Zheng, J.J., Moon, R.T., and Xu, W. (2008) Crystal structure of a full-length beta-catenin. *Structure* **16**, 336–338