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Effects of epitope sequence tandem repeat and proline incorporation on polyclonal antibody production against cytochrome 1A2 and 3A4

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We describe a method for producing polyclonal antibodies against peptide antigen cytochrome P450 1A2 and 3A4 using a tandem repeat of the epitope region and incorporation of proline residue between the repeated sequences. An ELISA assay revealed more efficient generation of polyclonal antibodies to tandem repeat peptide antigens than mono-epitope peptides. The incorporation of proline residues further stimulated antibody production. [BMB reports 2009; 42(7): 418-420]

INTRODUCTION

Both natural and recombinant proteins have been used to produce polyclonal antibodies (PAbs). However, natural protein antigens are rarely available in a pure form and recombinant proteins also have limitation to be used as ideal antigens due to the expression in host cells, correct folding problems, and purification processes.

In contrast, synthetic peptides are available in a highly pure form, which makes them useful to produce antibodies against proteins that have not been purified and can be identified only by cDNA or EST clones (1). In addition, peptide antigens can target the specific region(s) of the whole protein and may reduce the cross-reactivity of the antibodies produced with other antigens due to minimal epitope numbers. However, the wide use of peptide antigens has been constrained by difficulty of epitope region prediction from the whole protein sequence, antigen preparation concerns including peptide synthesis, purification, and conjugation to a carrier protein, and the relatively low antigenicity related to peptide length and choice of peptide sequence.

Presently, we report on the efficient production of PAbs against peptide antigens using a tandem repeat of the epitope sequence and incorporation of proline (Pro) residue as a spacer between the repeated regions.

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RESULTS AND DISCUSSION

The peptide antigens of cytochrome P450 1A2 (CYP1A2) and 3A4 (Table 1) have been established as epitopic regions (2, 3). We previously reported that the peptide sequences can be used to produce PAbs in rabbits when conjugated with a polymer carrier (4). Presently, we compared the effect of a tandem repeat of the epitope regions on antibody production.

Both tandem repeat peptides of the epitope regions from CYP1A2 and 3A4 (1A2-TR and 3A4-TR, respectively, in Table 1) were 2.2-2.6 fold more effective in the formation of PAbs compared to mono-epitope peptides (1A2-M and 3A4-M) when assayed with antiserum after antigen injection (Fig. 1A). The prepared antibody from the tandem peptides of CYP1A2 and 3A4 specifically recognized the corresponding proteins in human liver microsomes (Fig. 1B). These results imply that the repeated epitope peptide stimulates the increased production of PAbs as compared to the conventional epitope sequence. A definitive conclusion will require the testing of other peptide antigens to confirm the effect of tandem repeat sequence on PAb formation.

Table 1. Amino acid sequences of the peptide antigens used in this study

	Peptide	Amino acid sequence	Reference
CYP1A2	1A2-M	SENWKDN	(2)
	1A2-TR	SENWKDNSENWKDN	This study
	1A2-TR/P	SENWKDN**P**SENWKDN	This study
	1A2-TR/PP	SENWKDN**PP**SENWKDN	This study
	1A2-TR/A	SENWKDN**A**SENWKDN	This study
	1A2-TR/AA	SENWKDN**AA**SENWKDN	This study
	1A2-TR/G	SENWKDN**G**SENWKDN	This study
	1A2-TR/GG	SENWKDN**GG**SENWKDN	This study
CYP3A4	3A4-M	LEDTQKH	(3)
	3A4-TR	LEDTQKH LEDTQKH	This study
	3A4-TR/P	LEDTQKH**P** LEDTQKH	This study
	3A4-TR/PP	LEDTQKH**PP**LEDTQKH	This study

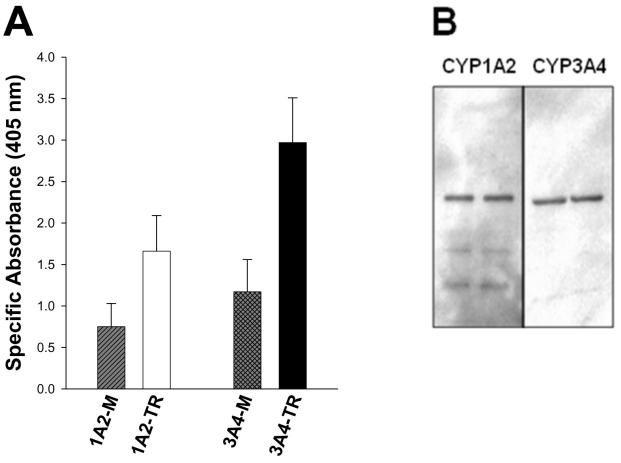

Fig. 1. Effect of the tandem repeat of peptide sequence on the amount of antibody produced and Western blotting using antibodies against the tandem repeat peptide. (A) After sampling the blood from immunized mice after antigen injection, serum samples diluted by 2,000 fold were analyzed by a conventional ELISA method using the peptide-BSA conjugate as an antigen as described in 'MATERIALS AND METHODS.' The anti-mouse IgG peroxidase conjugate and 2,2'- azino-di-(3 ethylbenzthiazoline sulfonic acid) were used as a secondary antibody and peroxidase substrate, respectively. Nonspecific binding of antigen to the wells without antigen immobilization was subtracted. (B) Western blot analysis of human liver microsomes using anti-sera prepared from the tandem repeat peptides of CYP1A2 and 3A4. The blots were developed by ECL™ according to a conventional method. Duplicate samples of microsomal proteins (80 μg for the detection of CYP1A2 and 40 μg for CYP3A4) were used for immunoblotting.

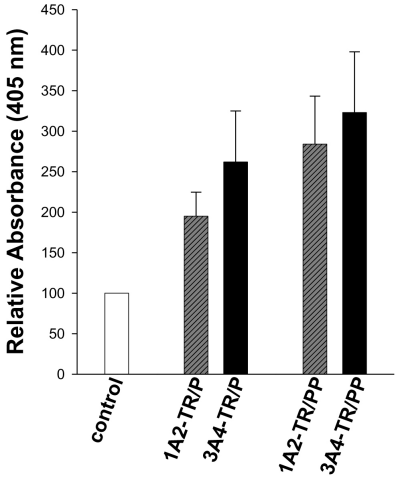

Fig. 2. Effect of incorporation of Pro residues in CYP1A2 and 3A4 epitope peptides on antibody production. Peptide antigen was synthesized to contain one or two Pro residues between the repeated epitope sequences and the amounts of antibody produced were compared with tandem repeat peptides (control: 1A2-TR and 3A4-TR). All the experimental conditions were the same as described in Fig. 1. The absorbance in ELISA assay using 1A2-TR peptide was set to 100% as a control.

To investigate the influence of the distance between the epitope regions on their antigenicity, a Pro residue was incorporated between the repeated sequences and the quantity of antibodies produced were compared to the spacer-free tandem repeat. Incorporation of one Pro residue stimulated antibody production, which the absorbance of enzyme-linke immunosorbant assay (ELISA) increased by approximately 2.0-2.5 fold when the spacer-free control was set to 100% (Fig. 2). This result suggests that the inclusion of the Pro residue improves the antigenicity of the CYP1A2 and 3A4 repeated epitope sequences, although the precise role of Pro in the design of epitope peptides is not clear at present. As well, it is not presently clear how the Pro residues stimulate antibody production of CYP1A2 and 3A4 peptide antigens, and whether the amino acid may induce another antigenic region in the tandem repeat sequences. Interestingly, incorporation of two Pro residues induced even more antibody production, with an increased absorbance of 2.8-3.2 fold as compared to the spacer-free control. Further incorporation of Pro residues could not be tested due to the difficulty in synthesizing the peptide and in purification.

On the basis of the results described in Figs. 1 and 2, we sought to relate the functional role of Pro residues with peptide antigenicity from the viewpoint of secondary structure of peptides, because proline has been known as a helix-breaking amino acid in α-helical regions of polypeptides. For example, using network protein sequence analysis (5) it was predicted that 3A4-TR peptide contains high content of α-helix and contrarily 3A4-TR/P and 3A4-TR/PP peptides have almost all random structure, which are probably due to the presence of Pro residues in the peptide sequence. Therefore, the current study seems to contradict previous reports suggesting that peptide antigenicity may be enhanced by helix stabilization in the C-terminal peptide of chicken riboflavin carrier protein (6, 7). However, many peptide antigens contain Pro residue(s) in epitope sequences that have a potential helix structure. Therefore, further systemic experiments need to be performed to clarify the relationship between antigenicity and secondary structure of peptide antigens.

To examine the effect on the antibody production of other amino acid residues used as a spacer between tandem repeat sequences, alanine (Ala) or glycine (Gly) residues were incorporated in the repeat sequence of CYP1A2 instead of Pro and the same assays were repeated. The use of Ala residues as a spacer had little effect on the increase in specific antibody formation, whereas incorporation of Gly residues, which has been also known as a helix-breaking amino acid, significantly decreased the absorbance in ELISA assay implying decrease in the antigenicity of the peptide (Fig. 3). Although other amino acids were not tested as a spacer in peptide antigens, our observations suggest that Pro residue(s) specifically assumes an important role in stimulating the antigenicity of the tandem repeat peptide.

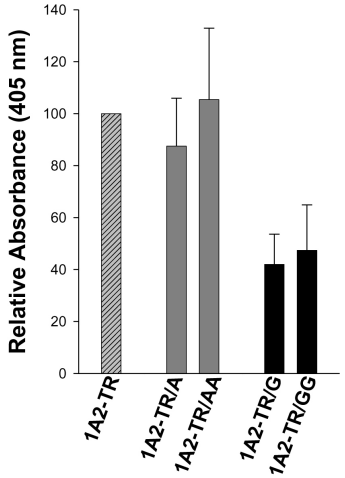

Fig. 3. Antibodies produced by the peptide antigens of CYP1A2 containing Ala or Gly substitutions instead of Pro residue in the tandem repeat sequence. The experiment was performed under the same conditions described in Fig. 1.

MATERIALS AND METHODS

Materials

BALB/c mice (5 to 7 weeks old) were obtained from Samtako (Osan, Korea). Peptide antigens summarized in Table 1 were synthesized by PepTron (Daejon, Korea). Keyhole Limpet Hemocyanin (KLH) was purchased from Pierce Biotechnology (Rockford, IL). Human liver microsome was purchased from BD Biosciences (San Jose, CA).

Conjugation between peptides and KLH

Peptides were conjugated with KLH using glutaraldehyde as a cross-linker (8). The molar ratio of KLH : peptide antigens was 1 : 100. KLH and peptide were dissolved in 2 mL of 0.01 M phosphate buffer (pH 7.4). Glutaraldehyde (20 mM, 1 mL) was then added to the reaction sample to achieve peptide-KLH conjugation. The potentially unreacted glutaraldehyde was removed by dialysis against an excess volume of phosphate-buffered saline. Peptide concentrations were determined using a fluorescamine assay (9).

Immunization

The injection samples were prepared by mixing the peptide-KLH complex with complete or incomplete Freund's adjuvant as a 1 : 1 (v/v) emulsion. The injection volume for the immunization of each mouse was approximately 0.4 mL. The antigen was administered intradermally to the shaved back skin of a mouse (five mice per group) in four injections (100 μl/injection) using a standard disposable 28-gauge syringe. The animals were similarly boosted 2 weeks later. Blood samples were taken from the tail vein of each mouse (total bleeding volume of approximately 300 μl) 2 weeks after the final injection.

Western blot assay

Western blotting analysis was conducted with the use of human liver microsomes for the detection of cytochromes P450 1A2 (CYP1A2) and 3A4 (CYP3A4) proteins.

ELISA assay

The level of anti-peptide antibodies was also determined using a conventional ELISA method. Eeach peptide was first conjugated to activated bovine serum albumin (Imject Maleimide Activated BSA, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions, and the peptide-bovine serum albumin (BSA) complex was then used as an ELISA antigen.

Statistical analyses

Results were presented as mean ± S.E. of 5 independent experiments and Student's t testes were used.

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