学 位 論 文 の 要 旨

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学 位 論 文 名　G196 Epitope Tag System: a Novel Monoclonal Antibody, G196, Recognizes the Small, Soluble Peptide DLVPR With High Affinity

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論 文 内 容 の 要 旨

INTRODUCTION

Monoclonal antibodies (mAbs) are among the most frequently used tools in both basic science research and in clinical diagnosis and therapies. Identification of the target epitope is of critical importance in the characterization of a mAb. To this end, understanding antibody specificity at the amino acid level provides key information for understanding the specific interaction between antibodies and their epitopes. mAb/epitope pairs provide a powerful tool as anti-tag mAb/tags when specific antibodies for the protein of interest are not readily available. There are a broad range of applications of mAb/epitope pairs in experimental biology, ranging from human to yeast, including monitoring protein expression, tracking and localizing proteins at subcellular levels, protein purification, the analysis of protein topology, dynamics and interactions, and the analysis of structural and functional proteomics. The most commonly used and well-characterized anti-tag mAb/tags are commercially available and include M2/FLAG-tag (DYKDDDDK), 9E10/c-Myc-tag (EQKLISEEDL), and 12CA5/HA-tag (YPYDVPDYA).

Short epitope tags (8-10 amino acid residues) are advantageous for minimizing side effects on the structure and biological function of the fused target protein. However, each tag designed to date has unique disadvantages and all tags, whether large or small, occasionally interfere with the structure, biological activity and/or crystallization of the fused protein. Furthermore, epitope tags are known to be post-translationally modified by phosphorylation, glycosylation, and sulfation in cells, and these modifications increase the molecular mass of the fused protein and change gel mobility. In addition, it has been reported that epitope modification can abolish anti-tag mAb/tag recognition.

Here we describe a new family of tag derived from the epitope recognized by a highly specific mAb G196.

MATERIAL AND METHODS

Mouse mAb G196 was generated by immunizing mice with glutathione *S*-transferase (GST) protein bacterially expressed using the pGEX-2T vector. All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University and they were handled according to our institutional guidelines.

For epitope mapping, a fragment of the GST gene was prepared by polymerase chain reaction amplification (PCR) and the pGEX-6P-1 plasmid as the template. The primers contained the *Bam*HI and *Xho*I restriction sites. The amplified fragments were digested with *Bam*HI and *Xho*I, then cloned into the pET28a vector (Merck Millipore) digested with the same two enzymes. Permutation enzyme-linked immunosorbent (ELISA) assay analysis was performed using the biotinylated 11-aa peptide (biotin-SGSGSDLVPRG, single positions of the peptide (epitope sequence underlined) were substituted with the other 19 coded amino acids) synthesized by Mimotopes.

Isothermal titration calorimetry (ITC) was performed with a VP-ITC titration calorimeter (MicroCal) at 25°C. Calorimetric measurements were carried out with purified G196 Fab and the synthetic peptide GSDLVPRGS (Tufts University Core Facility). Binding parameters were determined using the Origin software package provided with the instrument by fitting the data to a single-site model.

The DNA sequences of the PCR-amplifying heavy and light chain genes were determined with an ABI 3100 automated capillary DNA sequencer (ThermoFisher Scientific). mAb G196 Fab was crystallized using the hanging drop vapor diffusion method. The crystals grew at 20°C in 15% PEG 20000, 100 mM sodium citrate (pH 5.0) over a period of one week. The crystals were briefly soaked in a cryoprotectant solution consisting of the reservoir solution plus 20% (v/v) glycerol before cryocooling. X-ray data were collected using an ADSC Q270 CCD detector at beam-line BL17A of the Photon Factory, Tsukuba, Japan.

To ascertain the possible versatility of G196-tag as a versatile fusion tag for scientific research, we generated HeLa cells (JCRB9004) and fission yeast expression constructs in which the G196-tag was fused to reporter proteins.

RESULTS AND DISCUSSION

By analyzing a series of C-terminal fragments of GST, we identified the minimal epitope as the five amino acid sequence DLVPR and clarified that the epitope contains four critical residues and one nonessential residue (Pro at position 4) under denaturing conditions. Replacement of either residue (Asp to Glu at position 1; Arg to Lys at position 5) did not salvage immunoreactivity. Western blot analysis revealed that these charged amino acids at opposite ends of the epitope are critical residues for G196 antibody binding under denaturing conditions.

Permutation ELISA analysis showed that Asp at position 1 can be replaced by Glu and the

flexible amino acids Gly and Ser, whereas Leu at position 2 can be changed to one of several hydrophobic amino acids (Ile, Met, Phe, Asn) but not to Val, and to the hydrophilic amino acid His. Val at position 3 can be exchanged with one of two hydrophobic amino acids (Ile, Ala), and also with the hydrophilic amino acid Thr. Pro at position 4 does not show interaction specificity, whereas Arg at the last position is the most specific: Arg can not be substituted with Lys.

ITC indicated that a substantial exothermic reaction (change in enthalpy $\Delta H = -15.35$ kcal mol^{-1}) was observed upon mixing, resulting from a high-affinity binding event (dissociation constant $K_d = 1.25 \pm 0.77$ nM) with 1:1 stoichiometry ($n = 1.2 \pm 0.05$).

The structure of G196 Fab was determined as the antigen-free form at 2.0 Å resolution using the molecular replacement method. The Fab fragment displays a conventional immunoglobulin fold characterized by an anti-parallel β-sheet sandwich architecture (Protein Data Bank code 1IGY). Trp99 in complementarity-determining region (CDR)-H3 interacts with His35 in framework region (FR)-H2 through π-π aromatic stacking and forms hydrophobic core with Tyr32 and Trp33 in CDR-H1, and Phe103 in CDR-H3. Asn-31 in CDR-H1, Asn52 and Asn55 in CDR-H2, are located at one side of the hydrophobic core.

FLAG- and HA-tagged Emerald GFP (hereafter abbreviated FHG) fused with the G196-tag (FHG-G196) was detected as a single band in cell extracts by Western blotting with both anti-FLAG and the G196 mAbs, whereas, as expected, the reporter without G196-tag (FHG) was not detected by mAb G196. It is noteworthy that mAb G196 did not cross-react with cellular proteins, and reacted solely with the G196-tagged protein. In addition, mAb G196 efficiently immunoprecipited FHG fused with the G196-tag from cell extracts, comparable to that of FLAG immunoprecipitates, but did not immunoprecipitate FHG lacking the G196-tag. Immunofluorescence assays with HeLa cells showed that mAb G196 successfully detected the nuclear reporter protein (NAC1), as did the control polyclonal anti-GFP antibody. Western blotting showed that mAb G196 detected the Atf1 protein in yeast cell extracts, and chromatin immunoprecipitation (ChIP) enriched the Atf1 protein at the promoter of the *tdh1* gene, which harbors a CRE consensus site. Furthermore, mAb G196 recognized G196- and GFP-tagged Atf1, as shown by immunofluorescent staining of yeast, comparable to that of a polyclonal anti-GFP antibody.

These results indicate that the G196 epitope tag system is suitable for Western blotting, immunoprecipitation, ChIP, and immunofluorescence assay in both yeast and human.

CONCLUSION

We generated a mAb, named G196, by immunizing mice with GST protein bacterially expressed using the pGEX-2T vector, and identified that the minimal epitope of the mAb is the five amino acid sequence DLVPR. The mAb G196/G196-epitope interaction was characterized by permutation ELISA analysis, ITC, and structural analysis. The new G196 epitope tag system will thus be useful for a broad range of studies in cell biology and biochemistry.