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Epitope Mapping of an Anti-CD20 Monoclonal Antibody (C_{20}Mab-60) Using the HisMAP Method

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CD20 is one of the B-lymphocyte antigens and an effective target for the detection and treatment of B cell lymphomas; specific and sensitive monoclonal antibodies (mAbs) are required thus for their diagnosis. Recently, we developed a novel anti-CD20 mAb (clone C_{20}Mab-60), which is not only useful for flow cytometry but also for Western blot and immunohistochemical analyses. However, the epitope of C_{20}Mab-60 has not been determined. To clarify the binding region of mAbs against their target molecules, it is essential to understand the pharmacological function of each mAb. In this study, we aimed to identify the epitope of C_{20}Mab-60 for CD20 using the novel histidine tag (His-tag) insertion for epitope mapping (HisMAP) method. We first established an anti-His-tag mAb, HisMab-1 (mouse IgG_{2b}, kappa), by immunizing mice with recombinant proteins containing an N-terminal His-tag. Although HisMab-1 detected the 4x, 5x, and 6xHis tag-inserted CD20 proteins using flow cytometry, 5xHis tag was selected. While HisMab-1 recognized all the 5xHis tag-inserted CD20 from the 142nd to the 183rd amino acid (aa), C_{20}Mab-60 did not react with the 5xHis tag-inserted CD20 from the 171st to the 174th aa. These results indicate that the main epitope of C_{20}Mab-60 for CD20 is a peptide from 171st to 174th aa of CD20. HisMAP method could be advantageous in the determination of the critical epitope of functional mAbs against many target molecules.

Keywords: CD20, C_{20}Mab-60, epitope mapping, monoclonal antibody, His tag

Introduction

CD20 is a transmembrane protein containing four transmembrane domains, and consists of 297 amino acids (aa).[1] CD20 is expressed on B cells from pre-B to mature B cell development, and is also detected in many types of non-Hodgkin lymphoma,[2–4] implicated in B cell activation, differentiation, and regulation of calcium influx.[5,6] CD20 is expressed in 50% of B lymphoblastic leukemia/lymphoma originating from pre-B cells, but it is not detected in terminally differentiated plasma cell malignancies.[7,8] CD20 has been studied as a therapeutic target of B cell lymphomas and autoimmune disorders.[9–11]

Specific and sensitive monoclonal antibodies (mAbs) are critical for the diagnosis of many types of cancer. Using the Cell-Based Immunization and Screening method, we have successfully produced a specific and sensitive anti-CD20 mAb (clone C_{20}Mab-60) that can be used not only for flow cytometry but also for Western blot and immunohistochemical analyses.[12] The epitope of C_{20}Mab-60 has not been, however, elucidated.

In general, the epitope consists of several amino acids; mAbs often might therefore cross-react with other proteins besides their epitope. Determination of the epitope is important to avoid unexpected cross-reactivity and is crucial in the development of antibody drugs. Several methods have been previously reported for the characterization of epitopes, including X-ray cocrystallography, array-based oligo-peptide scanning, and site-directed mutagenesis mapping.[13]

The array-based oligopeptide scanning and site-directed mutagenesis mapping are useful for determining linear epitopes, but are not suitable for determining conformational epitopes. In contrast, X-ray cocrystallography can accurately determine epitopes as direct visualization of the interaction between the antigen and antibody can be achieved; however, the crystallization of the antigen–antibody complex usually

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takes a lot of time and is expensive. The development of simple and efficient methods for determining conformational epitopes would thus be crucial.

We previously developed a novel epitope mapping method named as the pentapeptide Arg79, Ile80, Asp81, Glu82, and Leu83 (RIEDL) insertion for epitope mapping (REMAP)[14,15] using a RIEDL tag system.[16] REMAP method is a simple and an efficient method for linear and conformational epitopes. In this study, we developed a histidine tag (His-tag) insertion for epitope mapping (HisMAP) method, aiming to determine the epitope region of C_{20}Mab-60 for CD20.

Materials and Methods

Plasmid preparation

DNA encoding the CD20 gene (IRAL012D02) was provided by RIKEN BRC through the National BioResource Project of MEXT, Japan. The open reading frame of CD20 was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Insertions of 4xHis-tag, 5xHis-tag, and 6xHis-tag (4xH*, 5xH*, and 6xH*) between Pro169 and Ala170 of CD20 sequence were performed using the HotStar HiFidelity Polymerase Kit (Qiagen, Inc., Hilden, Germany) with oligonucleotides containing His-tag insertions, resulting in the production of CD20–P169_4xH*_A170, CD20–P169_5xH*_A170, and CD20–P169_6xH*_A170 plasmids.

Insertions of 5xH* in the extracellular region of CD20 were performed using the HotStar HiFidelity Polymerase Kit with oligonucleotides containing 5xH* insertions at the selected position. Lys142_5xH*_Ile143 (K142_5xH*_I143) was produced for instance by inserting the 5-histidine sequence between Lys142 and Ile143 of CD20. Polymerase chain reaction fragments bearing the desired mutations were inserted into the pCAG-Ble vector using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan). The 5xH* insertion mutants produced were the following: K142_5xH*_I143, I143_5xH*_S144, S144_5xH*_H145, H145_5xH*_F146, F146_5xH*_L147, L147_5xH*_K148, K148_5xH*_M149, M149_5xH*_E150, E150_5xH*_S151, S151_5xH*_L152, L152_5xH*_N153, N153_5xH*_F154, F154_5xH*_I155, I155_5xH*_R156, R156_5xH*_A157, A157_5xH*_H158, H158_5xH*_T159, T159_5xH*_P160, P160_5xH*_Y161, Y161_5xH*_I162, I162_5xH*_N163, N163_5xH*_I164, I164_5xH*_Y165, Y165_5xH*_N166, N166_5xH*_C167, C167_5xH*_E168, E168_5xH*_P169, P169_5xH*_A170, A170_5xH*_N171, N171_5xH*_P172, P172_5xH*_S173, S173_5xH*_E174, E174_5xH*_K175, K175_5xH*_N176, N176_5xH*_S177, S177_5xH*_P178, P178_5xH*_S179, S179_5xH*_T180, T180_5xH*_Q181, Q181_5xH*_Y182, and Y182_5xH*_C183.

Cell lines

Chinese hamster ovary (CHO)-K1 and P3U1 cells were obtained from the America Type Culture Collection (ATCC, Manassas, VA). CHO/CD20 was produced in our previous study.[17] CD20 mutation plasmids were transfected into CHO-K1 cells using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants of CHO/CD20–P169_4xH*_A170, CHO/CD20–P169_5xH*_A170, and CHO/CD20–P169_6xH*_A170 were selected using a cell sorter (SH800; Sony Biotechnology Corp., Tokyo, Japan). CHO-K1 cells and transfectants were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere containing 5% CO_2. The transfectants were cultivated in a medium containing 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA).

Establishment of an anti-His-tag mAb

Female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under pathogen-free conditions. All animal experimentation procedures were approved by the Animal Care and Use Committee of Tohoku University. Two mice were immunized with N-terminal His-tagged SARS-CoV-2 spike protein (S2 subunit) (cat. no. 230-01103; RayBiotech Life, Inc., Norcross, GA) and 50 μg/mouse of using Imject Alum (Thermo Fisher Scientific, Inc.) to develop an anti-His-tag mAb, although our initial goal was to develop the anti-S2 spike protein of SARS-CoV-2 (www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm#SARS-CoV-2). The procedure included three additional immunizations with SARS-CoV-2 spike protein (50 μg/mouse), followed by a final booster injection of SARS-CoV-2 spike protein (50 μg/mouse) 2 days before harvesting splenic cells. Splenocytes were subsequently fused with P3U1 cells using polyethylene glycol 1500 (Roche Diagnostics, Indianapolis, IN). The hybridomas were then grown in RPMI-1640 supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, 5 μg/mL plasmocin (InvivoGen), and hypoxanthine/aminopterin/thymidine (Thermo Fisher Scientific, Inc.). N-terminal His-tag-positive wells were selected by Enzyme-Linked ImmunoSorbent Assay. After limiting dilution, HisMab-1 (IgG_{2b}, kappa), which can detect His-tag of N-terminus and C-terminus, was established.

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline, cells were treated with primary mAbs (1 μg/mL) for 30 minutes at 4°C and subsequently with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using a BD FACSLyric (Becton, Dickinson and Company, Franklin Lakes, NJ) for characterization of HisMab-1, or the EC800 Cell Analyzer (Sony Biotechnology Corp.) for HisMAP analyses.

Results

Characterization of HisMab-1 using flow cytometry analysis

Flow cytometry was performed using HisMab-1 against CHO/CD20–P169_4xH*_A170, CHO/CD20–P169_5xH*_A170, and CHO/CD20–P169_6xH*_A170. HisMab-1 recognized CHO/CD20–P169_4xH*_A170, CHO/CD20–P169_5xH*_A170, and CHO/CD20–P169_6xH*_A170 cells, but not parental CHO-K1 cells and CHO/CD20 cells

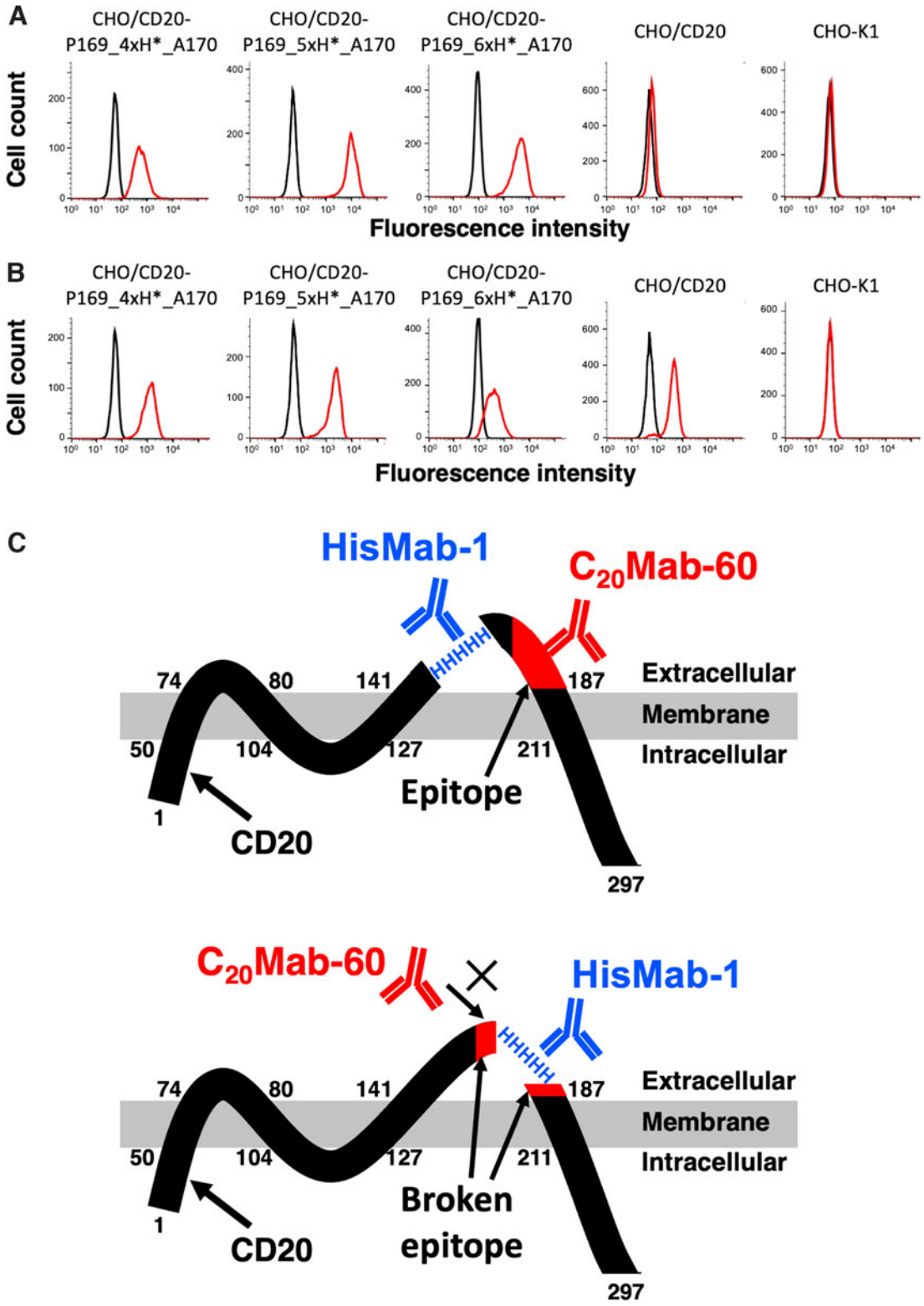

FIG. 1. Characterization of HisMab-1 using poly-histidine-inserted CD20. 4x, 5x, and 6xHis-inserted CD20 were expressed on CHO-K1 cells and then incubated with HisMab-1 **(A)** or anti-CD20 mAb (C_{20}Mab-11) **(B)** for 30 minutes at 4°C, followed by treatment with a secondary antibody. **(C)** C_{20}Mab-60 can bind to CD20 when 5xHis is inserted into any region, which is independent of the C_{20}Mab-60 epitope (upper panel). In contrast, C_{20}Mab-60 will not bind to CD20 when the conformation of the C_{20}Mab-60 epitope is disrupted by 5xHis insertion (lower panel). mAb, monoclonal antibody. H*, histidine.

(Fig. 1A). In contrast, an anti-CD20 mAb (clone C_{20}Mab-11[17]) that was used as a positive control reacted with all cells, except for parental CHO-K1 cells (Fig. 1B). These results indicate that HisMab-1 can detect 4xHis-tag, 5xHis-tag, and 6xHis-tag in flow cytometry analysis.

Determination of C_{20}Mab-60 epitope using HisMAP method

To investigate the C_{20}Mab-60 epitope, we conducted a His-tag insertion scanning, known as the HisMAP method. In

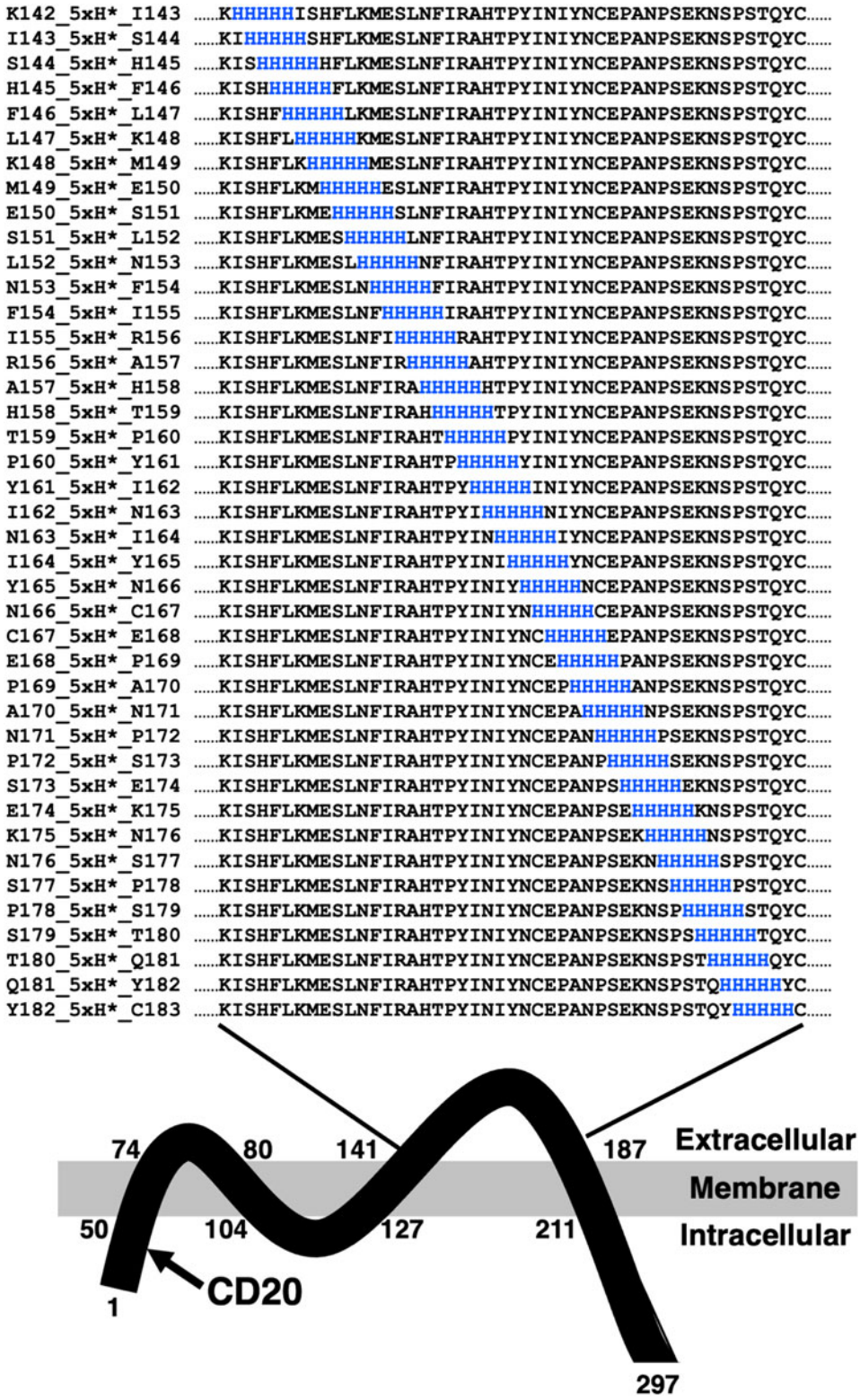

FIG. 2. Schematic illustration of HisMAP method. 5xHis was inserted into the expected epitope region within CD20. HisMAP, histidine tag insertion for epitope mapping.

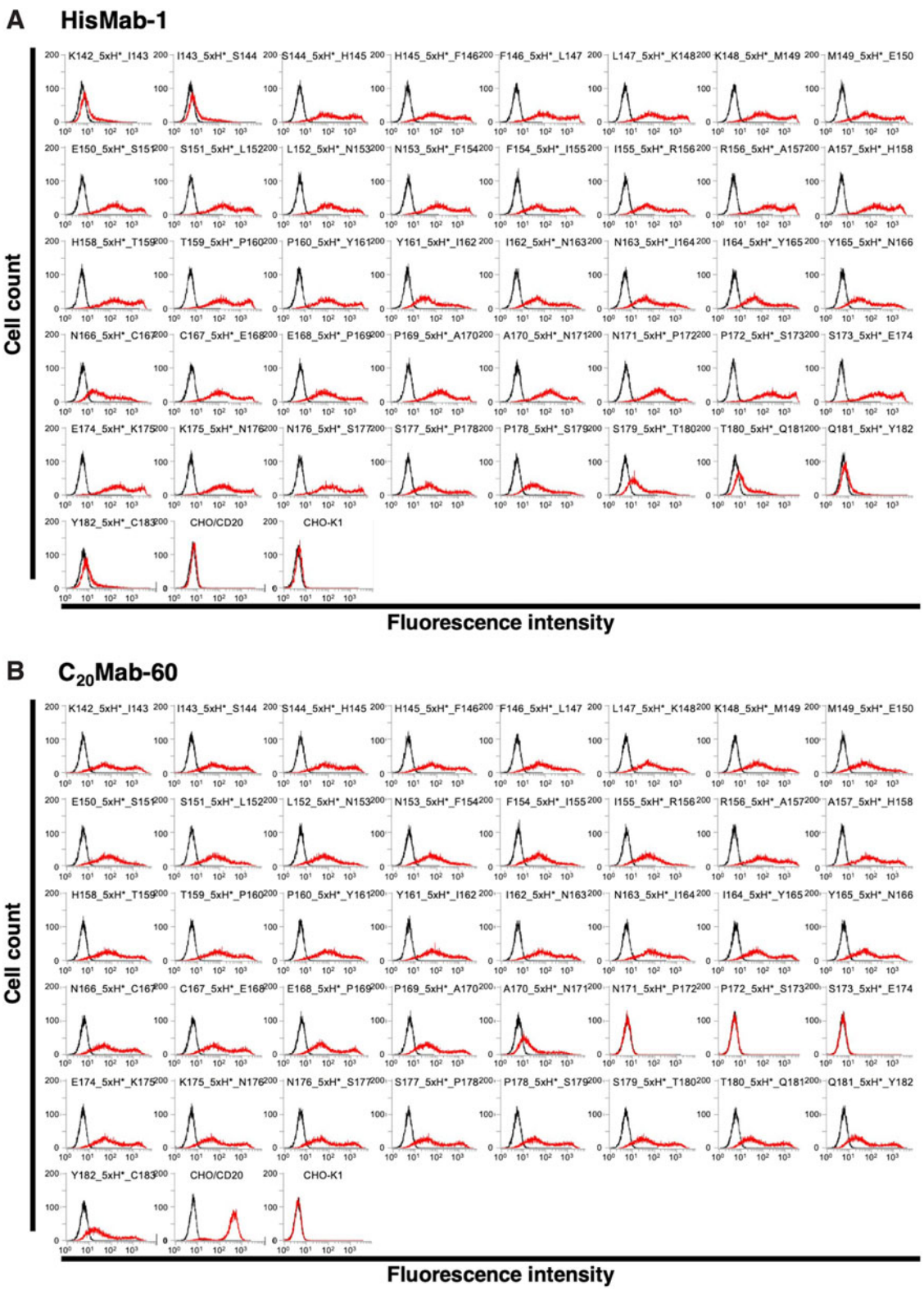

FIG. 3. Epitope mapping of C_{20}Mab-60 using His-tag insertion mutants of CD20. 5xHis-inserted CD20 mutants were analyzed using flow cytometry. Each mutant was expressed in CHO-K1 cells and incubated with HisMab-1 **(A)** or C_{20}Mab-60 **(B)** for 30 minutes at 4°C, followed by treatment with a secondary antibody. Red lines: treated with HisMab-1 or C_{20}Mab-60; black lines: no primary antibodies, used as negative controls. CHO-K1, Chinese hamster ovary-K1.

this method, we utilized a 5xHis-tag (5xH*) and an anti-His-tag mAb (clone HisMab-1). C_{20}Mab-60 can bind to CD20 when 5xH* is inserted in any region, independent of the C_{20}Mab-60 epitope (Fig. 1C, upper). In contrast, C_{20}Mab-60 did not detect CD20 when 5xH* is inserted into the C_{20}Mab-60 epitope region, while the conformation of the C_{20}Mab-60 epitope is disrupted by 5xH* insertion (Fig. 1C, lower).

We then constructed 41 mutants of CD20, in which 5xH* was inserted into the second extracellular loop region of CD20 (Fig. 2). Mutant proteins were transiently expressed on CHO-K1 cells, and analyzed using flow cytometry analysis. Results of the flow cytometry analysis showed that HisMab-1 used as a positive control detected all mutants (Fig. 3A). This result shows that all mutant proteins were expressed on CHO-K1 cells. Since 5xH* was not inserted in wild-type CD20, HisMab-1 did not react with CHO/CD20 (Fig. 3A). In contrast, C_{20}Mab-60 did not detect the three mutants (CHO/N171_5xH*_P172, CHO/P172_5xH*_S173, and CHO/S173_5xH*_E174) (Fig. 3B), indicating that the main epitope of C_{20}Mab-60 contains four amino acids (Asn171, Pro172, Ser173, and Glu174). These results are summarized in Figure 4.

Discussion

To identify the epitope of mAbs, it is important to avoid unexpected cross-reactivity, while it is essential to understand the pharmacological function of mAbs. Alanine-scanning mutagenesis and peptide screening are commonly used in the process of investigating new epitopes.[13,18–31] Although these methods are very useful in determining a linear epitope, a conformational epitope could not be determined by these methods. X-ray cocrystallography can accurately determine the epitope. Crystallization of the antigen–antibody complex, however, is costly and takes a lot of time. Development of a simple and efficient method for the determination of conformational epitopes would be thus essential.

Recently, we developed a novel epitope mapping method named REMAP method[14,15] using RIEDL tag system.[16] REMAP method is useful in determining conformational as well as linear epitopes.[14,15] In this study, we developed a novel epitope mapping system, named as HisMAP method. In the HisMAP method, we employed a His-tag, which is commonly used for protein purification and detection as an alternative of RIEDL tag.[32,33] Flow cytometry analysis showed that some 5xH* insertion mutants lose their reactivity to C_{20}Mab-60 (Fig. 3B). This result suggests that insertion of 5xH* causes partial disruption of the CD20 conformation and inhibits binding of C_{20}Mab-60 to CD20.

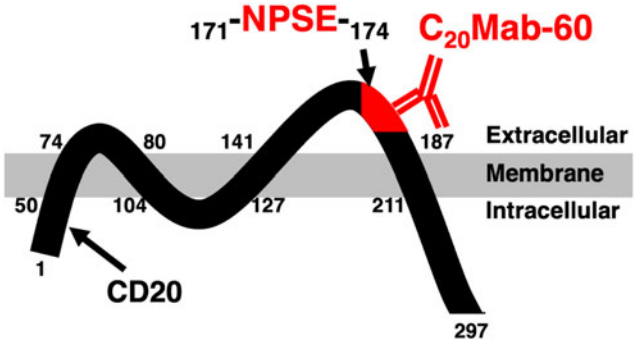

FIG. 4. Schematic illustration of epitope mapping of C_{20}Mab-60. Four amino acids (Asn171, Pro172, Ser173, and Glu174) are important for binding of C_{20}Mab-60 to CD20.

CD20 has two extracellular regions. The first loop between helix one and helix two is relatively small, and it is unlikely to protrude extensively, while the second loop between helix three and helix four consists of more than 40 amino acids.[34,35] We hypothesized thus that the epitope of C_{20}Mab-60 might be located at the second extracellular loop region of CD20. Using HisMAP method, we successfully determined that the critical epitope of C_{20}Mab-60 is located in Asn171, Pro172, Ser173, and Glu174 in the second extracellular loop region of CD20 (Fig. 4).

This work demonstrated that HisMAP method is useful as well as straightforward for epitope mapping of CD20. The epitope identification of C_{20}Mab-60 will be critical for the development of an antibody drug that targets CD20 in future studies. Interestingly, there are a number of mAbs for which the epitope has not been identified and could be characterized using the HisMAP method.

Author Disclosure Statement

No competing financial interests exist.

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