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Epitope Mapping of the Anti-CD20 Monoclonal Antibodies (C20Mab-11 and 2H7) Using HisMAP Method

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CD20, which is expressed on B lymphocytes, has been studied as a therapeutic target for B cell lymphomas and autoimmune disorders. Identifying the binding epitopes of monoclonal antibodies (mAbs) can contribute to our understanding of their functions. We have previously developed an anti-CD20 mAb (clone C_{20}Mab-11) using a Cell-Based Immunization and Screening (CBIS) method. In this study, we aimed to determine the binding epitopes of anti-CD20 mAbs, such as C_{20}Mab-11 and 2H7, using the His-tag insertion for epitope mapping (HisMAP). The results showed that $_{171}$-NPSE-$_{174}$ and $_{168}$-EPANPSE-$_{174}$ in the second loop of CD20 were essential for C_{20}Mab-11 binding and 2H7 binding, respectively. Although we developed many mAbs that recognize conformational epitopes using the CBIS method, there are many difficulties in epitope mapping for these mAbs. HisMAP could be useful for determining the conformational epitopes of other mAbs against membrane proteins.

Keywords: CD20, epitope mapping, monoclonal antibody, His tag

Introduction

CD20, a glycosylated transmembrane protein with two small and large extracellular loops (~7 and 44 amino acids), four transmembrane domains, and intracellular N- and C-terminal regions,[1,2] is expressed on normal B cells from pre-B to mature B cells, but not on pro-B cells and plasma cells.[3] CD20 is also detected in human B cell malignancies, including non-Hodgkin's lymphoma[4] and B lymphoblastic leukemia.[5] Since CD20 is not expressed on early pro-B cells, anti-CD20 monoclonal antibodies (mAbs), including rituximab, ofatumumab, and obinutuzumab, can destroy B cell malignancies through complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) without affecting the early lineage of normal B cells.[6–8]

Rituximab also affects CD20-expressing normal B cells and is approved for autoimmune disorders, including rheumatoid arthritis,[9,10] granulomatosis with polyangiitis,[11,12] and microscopic polyangiitis.[13,14] These mAbs have dramatically improved the prognosis of patients with CD20-positive malignancies and autoimmune diseases in the past two decades. Therefore, analyses of the specific nature of these mAbs, including their specific binding epitopes, affinities of target binding, and particular conformations, are important to understand their molecular activity. Epitope identification is particularly important for avoiding unexpected cross-reactivity and understanding the overall pharmacological function of mAbs. Nevertheless, determining the conformational epitopes of mAbs is challenging.

In our previous study, we established an anti-human CD20 mAb, C_{20}Mab-11 (mouse IgM, kappa), by using a Cell-Based Immunization and Screening (CBIS) method. C_{20}Mab-11 is useful not only for flow cytometry but also for Western blotting and immunohistochemical analyses.[15] In this study, we aimed to determine the binding epitopes of C_{20}Mab-11, developed by the CBIS method, and comparing it with a commercially available anti-human CD20 mAb (clone 2H7), using a His-tag insertion for epitope mapping (HisMAP).

Materials and Methods

Cell lines and antibodies

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

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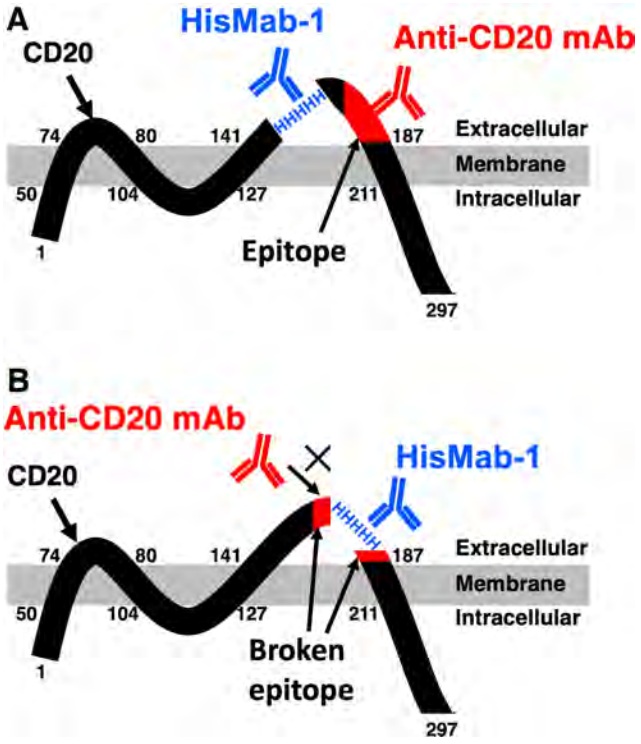


FIG. 1. Schematic illustration of HisMAP. The 5×H* is inserted into CD20. **(A)** C_{20}Mab-11 could bind to CD20 when 5×H* was inserted into any region, which was independent of the C_{20}Mab-11 epitope. **(B)** C_{20}Mab-11 did not bind to CD20 when the conformation of the C_{20}Mab-11 epitope was disrupted by the 5×H* tag insertion. HisMAP, His-tag insertion for epitope mapping.

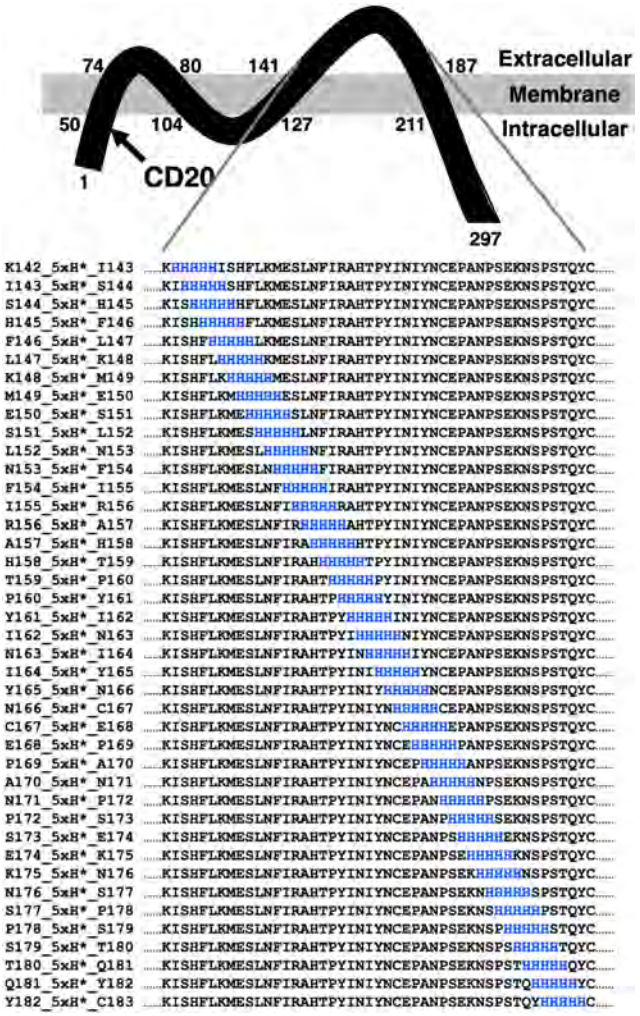


FIG. 2. The 5×H* tag was inserted into the large extracellular region of CD20.

study.[15,16] CD20 mutation plasmids were transfected into CHO-K1 cells using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). 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).

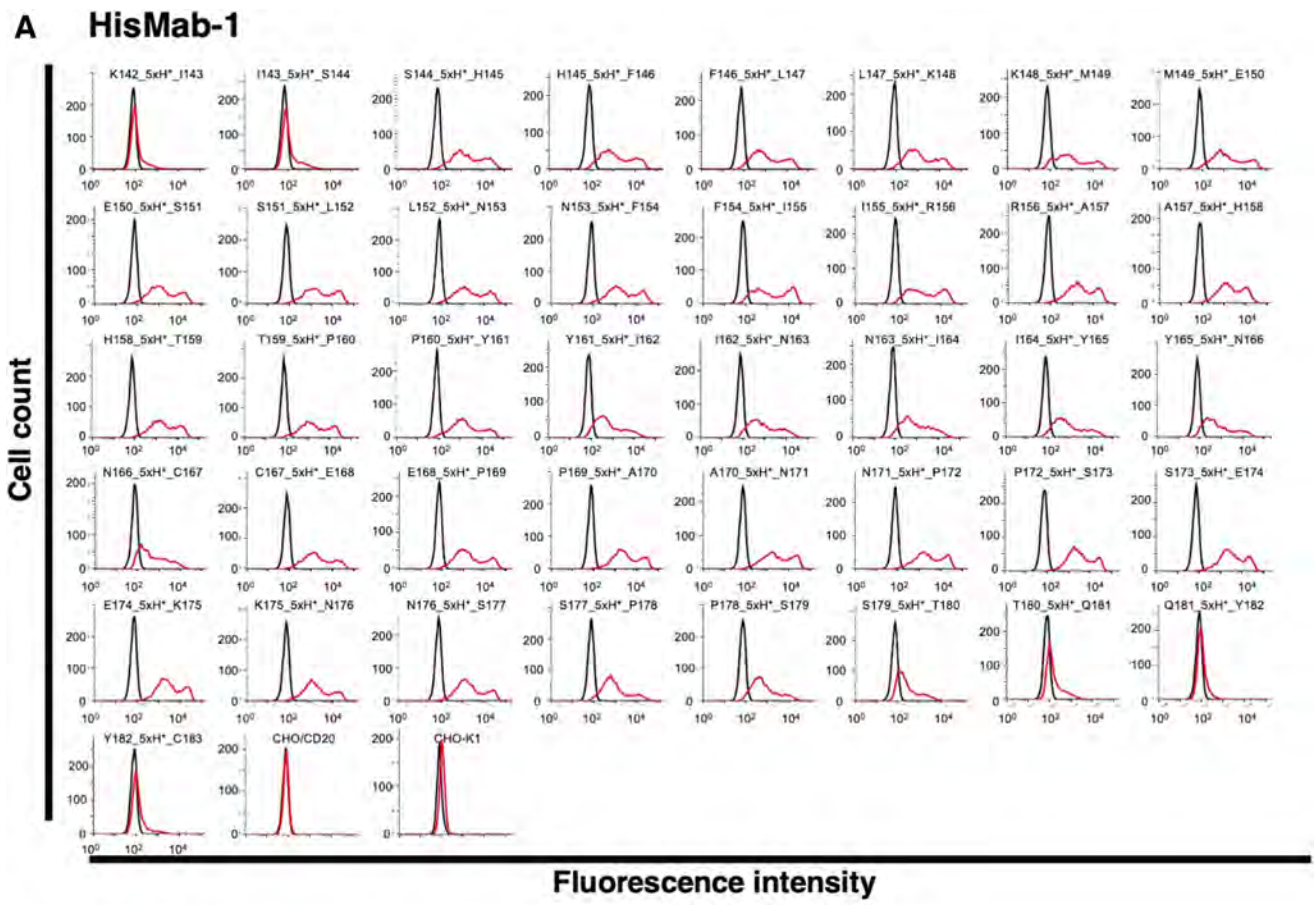
Anti-CD20 mAb (clone 2H7) was purchased from BioLegend (San Diego, CA).

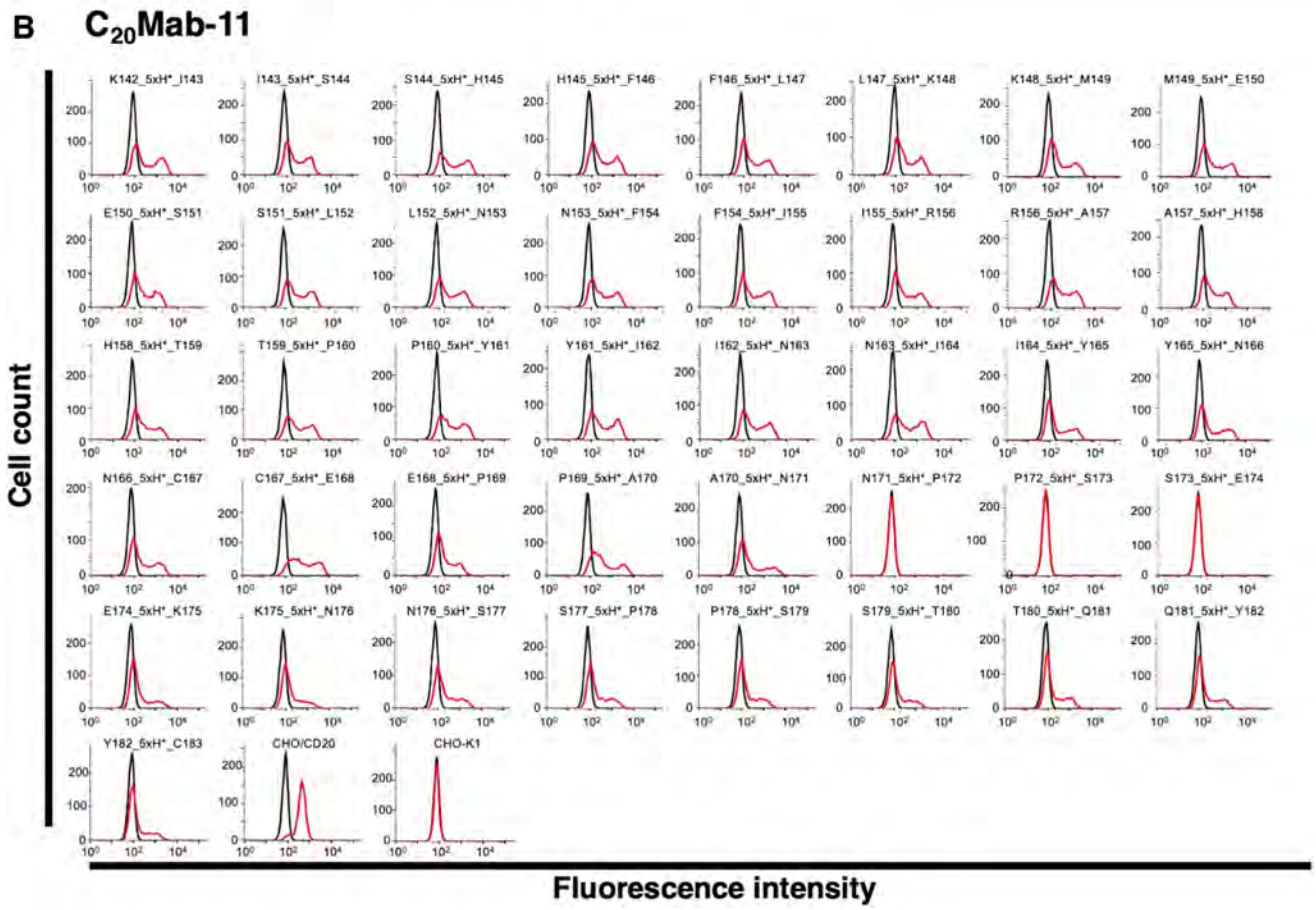
Establishment of an anti-His-tag mAb

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), 50 μg per 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 per mouse), followed by a final booster injection of SARS-CoV-2 spike protein (50 μg per 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 of streptomycin, and 0.25 μg/mL of amphotericin B, 5 μg/mL of plasmocin (InvivoGen), and hypoxanthine/aminopterin/thymidine (HAT; Thermo Fisher Scientific, Inc.). N-terminal

FIG. 3. Epitope mapping of C_{20}Mab-11 using 5×H* insertion mutants of CD20. The 5×H* inserted into CD20 and its mutants were analyzed using flow cytometry. Each mutant was expressed on CHO-K1 cells and incubated with HisMab-1 **(A)** or C_{20}Mab-11 **(B)** for 30 minutes at 4°C, followed by treatment with a secondary antibody. *Red lines*: treated with HisMab-1 or C_{20}Mab-11, *black lines*: without first antibodies as negative controls. CHO, Chinese hamster ovary.





His-tag-positive wells were selected by enzyme-linked immunosorbent assay. After limiting dilution, HisMab-1 (mouse IgG_{2b}, kappa), which can detect His-tag of N-terminus and C-terminus, was established.

Establishment of a recombinant anti-His-tag mAb

To produce recombinant HisMab-1, we subcloned V_H and C_H of cDNAs of HisMab-1 into the pCAG-Neo vector, along with V_L and C_L cDNAs of HisMab-1 into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. Vectors of HisMab-1 were transfected into ExpiCHO cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.). The resulting mAb (recHisMab-1) was purified using Ab-Capcher (ProteNova, Kagawa, Japan).

Plasmid preparation

DNA encoding the CD20 gene (IRAL012D02) was provided by RIKEN BRC through the National BioResource Project of MEXT, Japan.[15,16] The open reading frame of CD20 was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). Insertions of 5-histidine tag (5×H*) in the second loop of CD20 were performed using the HotStar HiFidelity Polymerase Kit with oligonucleotides containing 5×H* insertions at the selected position. Lys142_5×H*_Ile143 (K142_5×H*_I143) was produced for instance by inserting the 5-histidine tag 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 5×H* insertion mutants produced were the following: K142_5×H*_I143, I143_5×H*_S144, S144_5×H*_H145, H145_5×H*_F146, F146_5×H*_L147, L147_5×H*_K148, K148_5×H*_M149, M149_5×H*_E150, E150_5×H*_S151, S151_5×H*_L152, L152_5×H*_N153, N153_5×H*_F154, F154_5×H*_I155, I155_5×H*_R156, R156_5×H*_A157, A157_5×H*_H158, H158_5×H*_T159, T159_5×H*_P160, P160_5×H*_Y161, Y161_5×H*_I162, I162_5×H*_N163, N163_5×H*_I164, I164_5×H*_Y165, Y165_5×H*_N166, N166_5×H*_C167, C167_5×H*_E168, E168_5×H*_P169, P169_5×H*_A170, A170_5×H*_N171, N171_5×H*_P172, P172_5×H*_S173, S173_5×H*_E174, E174_5×H*_K175, K175_5×H*_N176, N176_5×H*_S177, S177_5×H*_P178, P178_5×H*_S179, S179_5×H*_T180, T180_5×H*_Q181, Q181_5×H*_Y182, and Y182_5×H*_C183.

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) or EC800 Cell Analyzer (Sony Biotechnology Corp.) for HisMAP analyses.

Results

Production of His-tag-inserted CD20 mutants

We conducted HisMAP to investigate the C_{20}Mab-11 epitope. C_{20}Mab-11 can bind to CD20 when 5×H* is inserted in any region independently on the C_{20}Mab-11 epitope (Fig. 1A). Contrastingly, C_{20}Mab-11 was not able to detect CD20 when 5×H* was inserted into the C_{20}Mab-11 epitope region because the insertion of 5×H* disrupted the conformation of the C_{20}Mab-11 epitope (Fig. 1B).

After 5×H* was inserted into the large extracellular loop region of CD20, 41 mutant plasmids of CD20 were constructed. The mutant plasmids were transfected and transiently expressed on CHO-K1 cells (Fig. 2).

Determination of C_{20}Mab-11 epitopes using HisMAP

Mutant protein expressing CHO-K1 cells were treated with HisMab-1 (Fig. 3A) or C_{20}Mab-11 (Fig. 3B) and were analyzed using flow cytometry. Flow cytometry showed that HisMab-1, used as a positive control, was detected in all mutants (Fig. 3A). Thus, all mutant proteins were expressed on CHO-K1 cells. HisMab-1 did not react with CHO/CD20 because 5×H* was not inserted into the wild type CD20 (Fig. 3A). Contrarily, C_{20}Mab-11 did not detect the three mutants (CHO/N171_5×H*_P172, CHO/P172_5×H*_S173, and CHO/S173_5×H*_E174) (Fig. 3B). Thus, four amino acids (Asn171, Pro172, Ser173, and Glu174) of CD20 were observed to be important for C_{20}Mab-11 binding (Fig. 4).

Determination of 2H7 epitope using HisMAP

Mutant protein expressed in CHO-K1 cells was treated with 2H7 (Fig. 5A), and analyzed using flow cytometry. Treatment with 2H7 was not able to detect the six mutants (CHO/E168_5×H*_P169, CHO/P169_5×H*_A170, CHO/A170_5×H*_N171, CHO/N171_5×H*_P172, CHO/P172_5×H*_S173, and CHO/S173_5×H*_E174) (Fig. 5A). Thus, seven amino acids (Glu168, Pro169, Ala170, Asn171, Pro172, Ser173, and Glu174) of CD20 were observed to be important for 2H7 binding (Fig. 5B).

Discussion

In this study, we determined the binding epitope of C_{20}Mab-11 and 2H7 as $_{171}$-NPSE-$_{174}$ and $_{168}$-EPANPSE-$_{174}$ in the second loop, respectively (Figs. 3–5). The epitopes

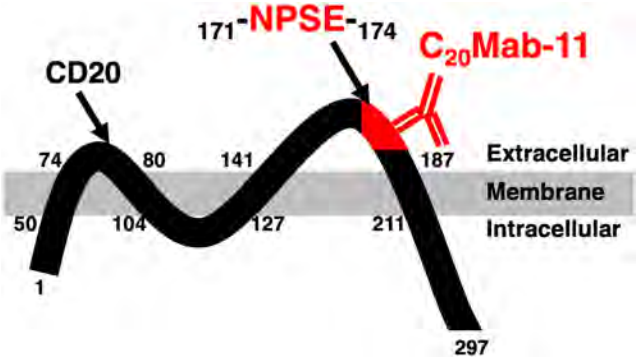


FIG. 4. Schematic illustration of epitope mapping of C_{20}Mab-11. Schematic illustration of C_{20}Mab-11 and its epitope. Four amino acids (Asn171, Pro172, Ser173, and Glu174) of CD20 were important for the binding of C_{20}Mab-11 to CD20.

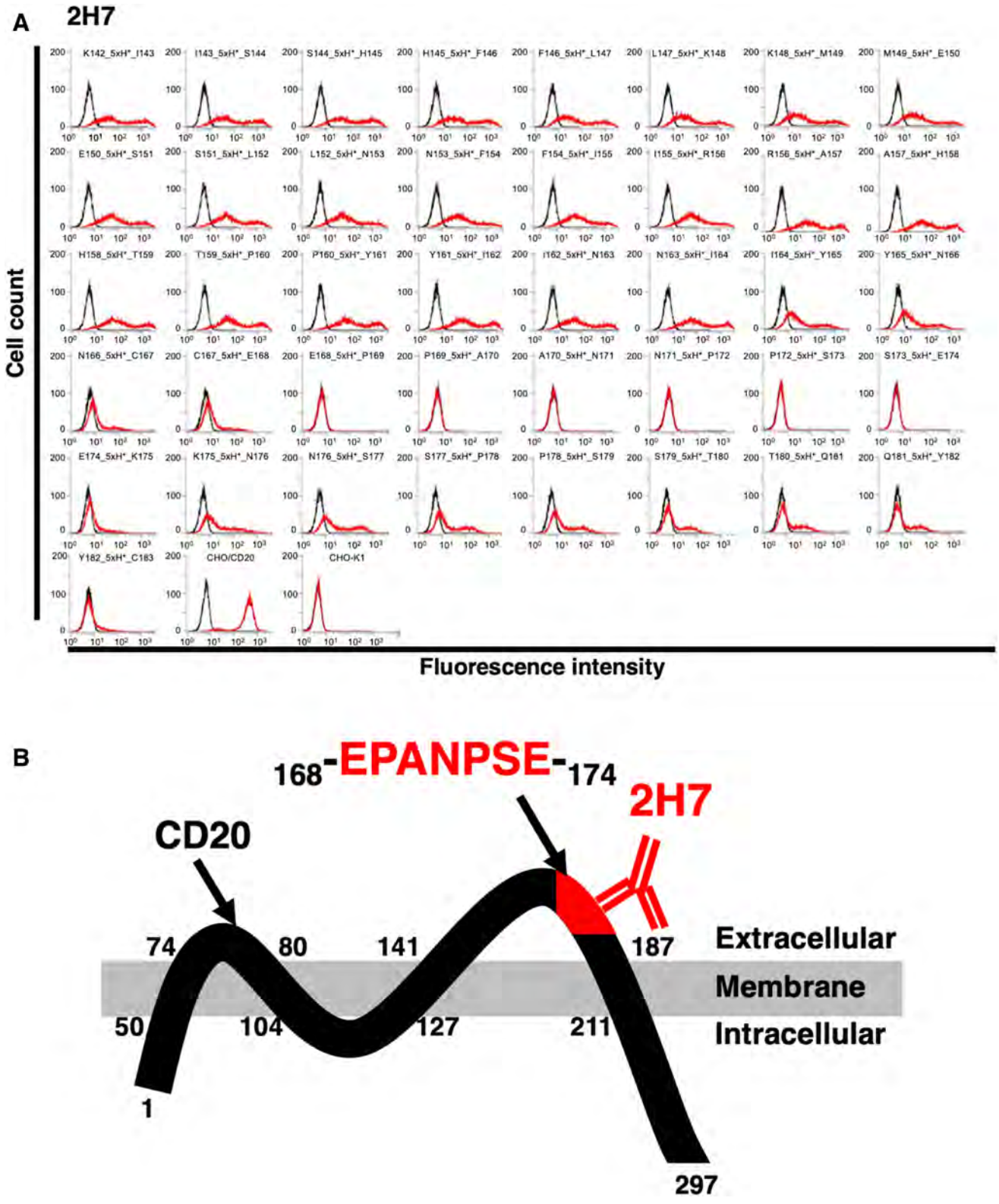


FIG. 5. **(A)** The 5×H* was inserted into CD20, its mutants were analyzed using flow cytometry. Each mutant was expressed on CHO-K1 cells and incubated with 2H7 for 30 minutes at 4°C, followed by treatment with a secondary antibody. *Red lines*: treated with 2H7, *black lines*: without first antibodies as negative controls. **(B)** Schematic illustration of 2H7 and its epitope. Seven amino acids (Glu168, Pro169, Ala170, Asn171, Pro172, Ser173, and Glu174) of CD20 are important for the binding of 2H7 to CD20.

of several anti-CD20 mAbs have been determined.[17–21] Rituximab recognizes a separated epitope of $_{170}$-ANPS-$_{173}$ and $_{182}$-YCYSI-$_{185}$ in the second loop.[17] $_{170}$-ANPSEKN-$_{176}$ is recognized by ocrelizumab,[18,19] obinutuzumab,[20] and C_{20}Mab-60.[21] Ofatumumab also recognizes a separated epitope of $_{72}$-IPAGIYAPI-$_{80}$, in the first loop, and $_{148}$-KMESLN FIRAHT-$_{159}$, in the second loop.[19] CD20 mAbs are classified into type I (CDC and ADCC) and type II (programmed cell death and ADCC).[8,22]

Type I mAbs, including rituximab and ofatumumab, stimulate the CD20 translocation into lipid rafts and potently activate CDC.[23] Both type I and II mAbs have ADCC. Type II mAbs, including obinutuzumab and tositumomab, can induce caspase-independent cell death.[24] Since the isotype of C_{20}Mab-11 is IgM, a class switch to IgG is required to elucidate the functions of C_{20}Mab-11. Furthermore, the identification of the complementarity-determining regions of C_{20}Mab-11 is essential in understanding the key structural features of its recognition to CD20.

We have developed two novel epitope mapping methods, a RIEDL insertion for epitope mapping (REMAP) and HisMAP, to determine the epitopes of anti-EGFR mAbs (EMab-51 and EMab-134)[25,26] and an anti-CD44 mAb (C_{44}Mab-46)[27] using the REMAP. The targets are type I transmembrane proteins and have conformational epitopes that cannot be determined by conventional methods, including deletion mutant and alanine scanning analyses. On the contrary, CD20 possesses four transmembrane domains and two extracellular loops (Fig. 1).

Previously, we reported that HisMab-1 can detect 4×H*, 5×H*, and 6×H* inserted into a target protein, using flow cytometry.[21] We further optimized HisMAP and determined that 5×H* insertion was minimally required. 5×H* was inserted into the large extracellular loop of CD20 (Fig. 2). Although the reactivity of HisMab-1 tended to decrease upon insertion of 5×H* close to the transmembrane domain (Fig. 3A), the epitopes of C_{20}Mab-11 and 2H7 were successfully determined. The 5×H* insertion did not affect the exposure of the extracellular loop of CD20 and, therefore, HisMAP could be applied to determine the epitopes of proteins with several transmembrane domains. Further studies are needed to reveal the usefulness of HisMAP for the determination of the conformational epitopes of the other mAbs.

Author Disclosure Statement

No competing financial interests exist.

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