Brief report

The epitope recognized by rituximab

Mascha Binder, Florian Otto, Roland Mertelsmann, Hendrik Veelken, and Martin Trepel

Rituximab is a monoclonal antibody widely used in the treatment of malignant lymphoma and autoimmunity. Its epitope within the B-cell antigen CD20 is largely unknown. We used phage display libraries to select peptides binding to rituximab. Enriched peptides showed 2 sequence patterns: one motif (CALMIANSC) is related to (170)ANPS(173) within CD20, while another motif (WEWTI) may mimic the CD20 segment (182)YCYSI(185). Phages displaying either motif specifically bound rituximab. Binding to rituximab by the CD20 peptides ANPS and YCYSI was weak when used separately and enhanced when both peptides were linked. Recombinant CD20 extracellular loop proteins blocked binding of the selected CWWEWTIGC phage to rituximab, suggesting that CWWEWTIGC mimics the epitope. Blocking capacity was strongly reduced upon mutation of the CD20 strings ANPS or YCYSI. We conclude that rituximab binds a discontinuous epitope in CD20, comprised of (170)ANPS(173) and (182)YCYSI(185), with both strings brought in steric proximity by a disulfide bridge between C(167) and C(183). (Blood. 2006;108:1975-1978)

Introduction

The CD20-targeted monoclonal antibody rituximab plays a major role in the treatment of B-cell non-Hodgkin lymphoma[1-3] and autoimmune disorders.[4] CD20 is a 33- to 35-kDa membrane protein on B cells with an extracellular loop (approximately 43 amino acids) containing the binding site of rituximab.[5] CD20 is a Ca^{2+} channel with unknown functional significance.[6,7] The mechanisms of rituximab-mediated B-cell depletion include cell- and complement-dependent cytotoxicity, induction of apoptosis, and sensitization to chemotherapy.[8-11]

There has been tremendous interest in identifying the epitope recognized by rituximab, as it may contribute to understanding rituximab cytotoxicity and may be useful for vaccine development in patients with lymphoma. The amino acid residues alanine[170] and proline[172] within the extracellular loop of CD20 are critical for rituximab binding.[12] Selection of random libraries yielded 2 distinct peptides binding rituximab[13,14]: one peptide was homologous to alanine (170)-proline(172), the other—although not homologous—was assumed to mimic the same epitope.[13,14]

Phage display peptide libraries can be used to identify antibody epitopes.[15-18] We used this approach to identify the epitope of rituximab. Our findings suggest a discontinuous epitope within the extracellular egment of CD20, consisting partially of the ANPS peptide and partially of the YCYSI peptide, both brought in steric proximity by a disulfide bridge.

Study design

Phage display

Phage peptide libraries were made as described for phage[19,20] and for plasmid libraries in adeno-asscociated viral genomes.[21] The degenerate insert was $TGT(NNB)_7TGT$. Libraries were screened on rituximab (Roche, Basel, Switzerland) basically as described.[22] In the third and fourth rounds of selection, the library was precleared on the antibody cetuximab (Merck, Darmstadt, Germany). Recovered clones were sequenced and tested for binding to rituximab or basiliximab (Novartis, Basel, Switzerland) as described.[22]

GST fusion proteins

Single-stranded oligonucleotides (Hermann, Denzlingen, Germany) were converted to double-stranded using the Sequenase Kit (Amersham, Arlington Heights, IL) and a suitable primer, subsequently digested with *Bam*HI and *Eco*RI, and ligased into pGEX-2TK (Amersham). Plasmids were transformed into BL21 bacteria (Stratagene, La Jolla, CA). Proteins were purified following Amersham's instructions.

Results and discussion

Phage library biopanning on rituximab yields specific peptides

A random CX_7C phage peptide library (C indicates cysteine; X indicates any amino acid) was selected on immobilized rituximab. The enrichment of phage on rituximab was monitored by counting transducing units recovered from the rituximab-coated versus cetuximab control wells. In the fourth selection round, phage bound 400 times stronger to rituximab than to cetuximab, indicating that rituximab-specific clones had been enriched. Sequencing of the inserts of 26 clones recovered from this round revealed the consensus motif W-W-E-W-[S or T] (Table 1).

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Table 1. Peptide insert sequences enriched after 4 selection rounds on immobilized rituximab using a random CX_7C phage display peptide library

Amino acid sequences, single letter code	Absolute frequency (%)
C W W E W T A T C	11 (42.3)
C W W E W T I G C	3 (11.5)
C W W E W T G A C	2 (7.7)
C Q F T V W E W C	1 (3.8)
C C L G R W C W C	1 (3.8)
C W E W S G N G C	1 (3.8)
C S W T S L P F C	1 (3.8)
C H A W M G L R C	1 (3.8)
C F K R S G S L C	1 (3.8)
C W R R G Y G G C	1 (3.8)
C K P W F P E L C	1 (3.8)
C A L M I A N S C	1 (3.8)
C R L T A N L P C	1 (3.8)

A total of 26 randomly picked clones were sequenced. Sequences are aligned for the consensus motif, which is indicated by underlined letters.

Phage clones specifically bind rituximab

The binding of enriched phages to rituximab was compared with binding to the isotype control basiliximab. All of the clones displaying the consensus motif but not insertless fd-tet control phages bound specifically to rituximab; none of the clones bound to basiliximab (Figure 1A).

The selected consensus motif W-W-E-W-S/T did not display obvious sequence homology to CD20 (Swissprot database; http://www.ebi.ac.uk/swissprot). Previous reports suggest that the CD20 peptide string (170)ANPS(173) may be important for rituximab binding.[12-14] One of our clones showed similarity to ANPS (CALMIANSC). This phage also specifically bound to rituximab (Figure 1A). This may imply that the CALMIANSC phage indeed mimics the ANPS-peptide string in CD20.

Sequence homology to CD20

Tyrosine and serine play a pivotal role in antigen-antibody interactions.[23,24] This may put our finding that our phage-derived W-W-E-W-S/T sequence is not homologous to CD20 into another perspective. The structure of a phage-displayed peptide is dictated by its way of presentation (eg, length or cyclization). Thus, modifications of the native epitope sequence such as the substitution of an amino acid by a slightly different equivalent may be a prerequisite for a phage-displayed peptide to structurally mimic an epitope. When analyzing phage-derived sequences, it may therefore be appropriate to consider functional classes of amino acids rather than single residues, and to focus on amino acids most relevant for protein interactions. Amino acids biochemically comparable to the aromatic tyrosine (eg, tryptophane) or to the polar serine (threonine) were abundant in the consensus motif recovered from our library selection on rituximab (Table 1). Therefore, we "translated" this motif by replacing tryptophane with tyrosine and threonine with serine. These "translated" sequences, in fact, revealed sequence homology to the peptide (182)YCYSI(186) within the extracellular loop of CD20 (Figure 1B). This homology, in conjunction with the binding data (Figure 1A), led us to hypothesize that both CD20 peptide strings, (170)ANPS(173) and (182)YCYSI(186), may be required for CD20 binding to rituximab.

Phage-displaying peptide strings of CD20 bind rituximab

To further explore whether both the ANPS and the YCYSI peptide strings play a role in CD20 binding to rituximab, we designed phages with 3 different peptides: (1) ANPS; (2) YCYSI; and (3) both strings within 1 peptide, separated by 8 neutral amino acids (G). Binding of these phages to rituximab was analyzed and compared with basiliximab binding. While there was low but detectable binding of the YCYSI clone to rituximab, stronger binding was observed for the ANPS clone and even stronger binding for the clone displaying both peptide strings (ANPS-$(G)_8$-YCYSI; Figure 2A). This substantiates the hypothesis that both the ANPS and the YCYSI peptide strings participate in the formation of the epitope recognized by rituximab.

Mutants of the CD20 extracellular loop interfere with binding to rituximab

Finally, we manipulated the CD20 protein and evaluated the consequences on rituximab binding. The aim of this was 2-fold: (1) to prove that the WWEWT motif truly mimics the CD20 epitope;

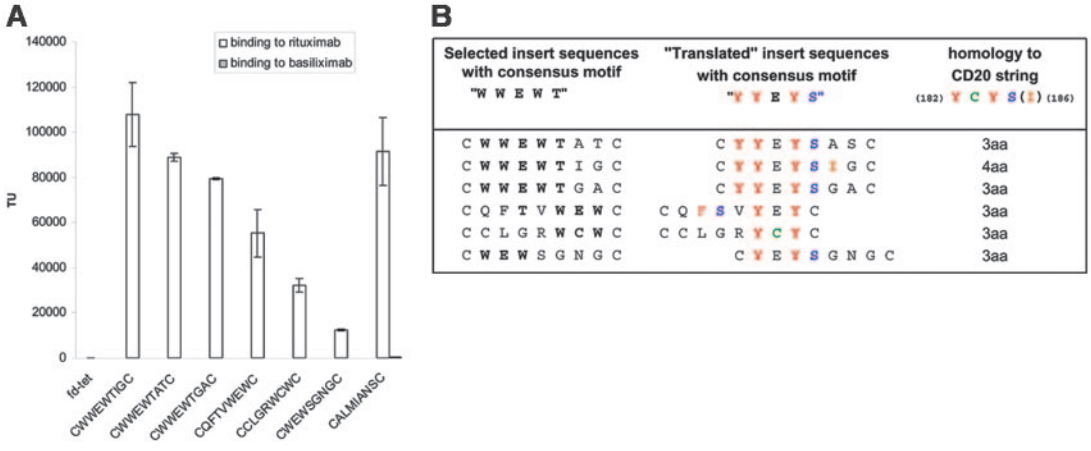

Figure 1. Selection of phage-displayed peptides binding to rituximab. (A) Phages displaying the enriched peptides specifically bind to rituximab but not to the control isotype antibody basiliximab. Phages (5×10^7 transducing units [TU]) displaying no peptide insert (fd-tet) or the peptides enriched by selection on rituximab were incubated on immobilized rituximab or the basiliximab control, respectively. Bound phages were recovered by K91 bacterial infection. Transduced bacteria were grown on LB plates containing tetracycline to determine the number of TUs by colony counting. Data are means from triplicate platings ± SEM. As binding is approximately 80 000 times stronger to rituximab compared with basiliximab, this background binding level is too low to be visible in the graph. (B) "Translated" insert sequences display sequence homology to the CD20 protein. The aromatic amino acids and the threonine within the enriched peptide sequences were replaced with tyrosine and serine, respectively. These translated sequences revealed sequence homology to the YCYSI string within the putative extracellular domain of CD20. aa indicates amino acids.

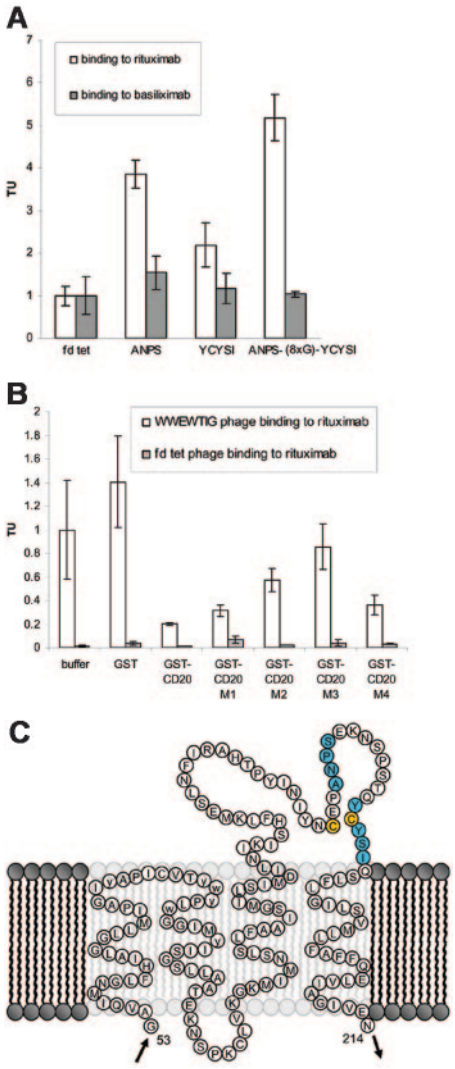

Figure 2. Rituximab binds to a discontinuous epitope within the extracellular domain of CD20. (A) Phages displaying both parts of the presumed epitope bind stronger to rituximab than phage displaying ANPS or YCYSI alone. Phages (5×10^7 TU) displaying no peptide insert (fd-tet) or the indicated peptides, respectively, were incubated both on immobilized rituximab and control antibody basiliximab. Bound phages were recovered by K91 bacterial infection. Transduced bacteria were grown on LB plates containing tetracycline to determine the number of TUs by colony counting. TU counts are shown relative to the number of TUs recovered from wells incubated with fd-tet. Data are means from triplicate platings ± SEM. (B) GST fusion proteins block binding of CWWEWTIGC phages to rituximab depending on their mutational status. CWWEWTIGC phages or insertless fd-tet phages (5×10^7 TU), respectively, were incubated on immobilized rituximab in the presence or absence of excess molar concentrations (0.2 mg/mL) of GST fusion proteins containing the presumed wild-type or mutated sequences shown in Table 2. Bound phages were recovered by bacterial infection. Transduced bacteria were grown on LB plates containing tetracycline to determine the number of TUs by colony counting. TUs are indicated relative to binding of WWEWTIG phages in the absence of GST fusion protein. Data are means from triplicate platings ± SEM. Phage binding is significantly blocked by GST-CD20 ($P = .018$), GST-CD20 M1 ($P = .023$) and GST-CD20 M4 ($P = .029$) compared with the GST control. (C) Model of the transmembrane and the extracellular domain of CD20 (based on Ernst et al[5]) with the suspected discontinuous epitope marked in blue. The disulfide bridge between C^{167} and C^{183} (Ernst et al[5]) is marked in yellow.

Table 2. Sequences of GST fusion proteins representing a 21–amino acid segment (aa 167–187) of the extracellular loop of CD20

Clone designation	Sequence of CD20 homolog fused to GST
GST-CD20	C E P A N P S E K N S P S T Q Y C Y S I Q
GST-CD20 M1	C E P A N P S E K N S P S T Q Y C G S I Q
GST-CD20 M2	C E P A N P S E K N S P S T Q G C G S I Q
GST-CD20 M3	C E P G N G S E K N S P S T Q Y C Y S I Q
GST-CD20 M4	C E P A N P S G K N S P S T Q Y C Y S I Q

Proteins with a mutation of the CD20 sequence are designated M1-M4. The amino acids considered to be important for rituximab binding were replaced by glycine (underlined letters). M4 had a mutation in a region adjacent but outside the ANPS peptide string, which is suspected to be critical for binding of rituximab.

and (2) to study the influence of mutating the ANPS or the YCYSI string on rituximab binding in order to elucidate the role both strings play in the formation of the epitope. Therefore, GST fusion proteins were designed displaying amino acids 167 to 187 of the CD20 extracellular loop in wild-type sequence or mutations thereof (Table 2). Binding of the CWWEWTIGC phage to immobilized rituximab was analyzed in the presence or absence of these proteins. The protein displaying the wild-type CD20 sequence blocked binding of the CWWEWTIGC phage to rituximab (Figure 2B), indicating that CWWEWTIGC truly mimics the CD20 epitope, as both seem to compete for rituximab binding. Mutant M1 had somewhat lesser blocking capacity, and both mutants M2 and M3 had markedly reduced blocking capacity (Figure 2B). This indicates that the structural similarity of the epitope homolog CWWEWTIGC and CD20 is lost upon mutation of the YCYSI or the ANPS peptide strings of CD20. This further substantiates that both peptide strings participate in the formation of the epitope recognized by rituximab.

The assumption that both parts of the presumed discontinuous epitope contribute to rituximab binding is supported by published data: rituximab binds a CD20 segment comprising amino acids 165 to 184. When the segment only comprised the previously identified critical residues alanine(170) and proline(172) but not tyrosine(182) and tyrosine(184), rituximab binding to the peptide is lost.[13] Moreover, a recently described disulfide bond between C(167) and C(183)[5] apparently brings both parts of the assumed epitope in steric proximity (Figure 2C). Consequently, reduction of the disulfide bond ablates CD20 binding to rituximab.[5]

Taken together, we conclude that the long-searched-for epitope recognized by rituximab is formed by the peptides (170)ANPS(173) and (182)YCYSI(186), brought together head to head by the disulfide bond C(167)-C(183).

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