Identification of a Cytoplasmic Region of CD20 Required for Its Redistribution to a Detergent-Insoluble Membrane Compartment

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Identification of a Cytoplasmic Region of CD20 Required for Its Redistribution to a Detergent-Insoluble Membrane Compartment

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CD20 is a B lymphocyte integral membrane protein with signal-transducing properties. Abs directed toward extracellular CD20 epitopes activate nonreceptor tyrosine kinases and modulate cell cycle progression of B lymphocytes. Recently, we demonstrated that binding of CD20Abs to B cells induces the rapid redistribution of up to 95% of CD20 molecules to low density, detergent-insoluble membrane microdomains and induces the appearance of an approximately 50-kDa tyrosine-phosphorylated protein in the same compartment. Active relocalization of CD20 may thus be critical to the initiation of signaling events by CD20. The CD20 cDNA sequence predicts a nonglycosylated protein with four transmembrane-spanning regions and intracellular amino and carboxyl termini. Here we provide verification of the location of both the intracellular and extracellular regions of the CD20 molecule and identify a membrane-proximal sequence in the cytoplasmic carboxyl tail that is required for CD20 to redistribute to detergent-insoluble membrane microdomains. The Journal of Immunology, 1998, 161: 3242–3248.

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d20 is a B cell membrane protein capable of transducing cell cycle progression signals in resting B cells upon Ab ligat

tion (1–5). Recently, we demonstrated that Ab binding to the CD20 extracellular domain causes rapid redistribution of a large majority of CD20 molecules to a low density, detergent-insoluble plasma membrane compartment (6). In many cell types this compartment includes caveolae, microinvasinations in the plasma membrane where Src family tyrosine kinases, G protein-coupled receptors, GPI-linked proteins, calcium channels, and ATPases, are found (7, 8). Although there is an apparent absence of caveolae in lymphocytes, biochemically similar structures can be isolated, sometimes referred to as rafts or DIGS (detergent-insoluble glycolipid-enriched structures) (9, 10). These microdomains are estimated to contain <1% of membrane proteins and, like caveolae, are enriched in a variety of proteins involved in signal transduction, including Src family tyrosine kinases, multiple heterotrimetric G proteins, and GPI-linked proteins (8). We have proposed that active relocalization of CD20 into these microdomains is a necessary event in the initiation of CD20 signaling, and in support of this, we find that an activating anti-CD20 mAb uniquely induces the appearance of a tyrosine-phosphorylated protein of about 50 kDa in the same compartment (6). There are only a few reports in the current literature of active relocalization of cell surface molecules into or out of detergent-insoluble domains or caveolae (11–16), and there is no information yet on the mechanism(s) that controls their redistribution. CD20 provides an excellent model for investigating the mechanisms involved in redistribution, since it is completely excluded from detergent-insoluble domains in the resting state and can be easily and massively induced to translocate into them upon Ab binding (6).

Human CD20 and its murine equivalent, Ly-44, have been cloned, and their genetic sequences have been characterized (17–20). Based on hydrophobicity data and the lack of a signal sequence, CD20 is predicted to have intracellular N- and C-termini, four transmembrane spans (TM1–4), and an extracellular domain between TM3 and TM4 (18, 19). We sought to dissect the cytoplasmic regions of CD20 in search of sequences involved in controlling its redistribution. However, since signal sequences are not always necessary for extracellular localization of N-terminal regions (21–26), the predicted membrane orientation of CD20 required confirmation.

In this study Abs generated against peptides in the N- and C-terminal regions of CD20 were used to determine its membrane orientation. Then, deletion mutants of intracellular regions were generated and examined to determine their effects on the redistribution of CD20 to Triton-insoluble membrane microdomains.

Materials and Methods

Cells and Abs

Raji lymphoblastoid B cells were grown in RPMI/5% FBS. Molt-4 T cells expressing transfected CD20 cDNA (27) were grown in RPMI/10% FBS in the presence of 0.4 mg/ml genetin (Life Technologies, Gaithersburg, MD). CD20-specific 2H7 mAb was provided by Dr. J. Ledbetter (Bristol-Myers Squibb, Seattle, WA). Antiserum against the amino and carboxyl regions of CD20 (herein named anti-CD20N and anti-CD20C) were generated by immunizing rabbits with either OVA-conjugated CD20N peptide (CD20N-P; residues 25–41, SGPKPLFRRMSSLVGPT) or OVA-conjugated CD20C peptide (CD20C-P; residues 231–245, SAEKKEQJTEI KEE; peptides were provided by James Blake, Bristol-Myers Squibb). The Abs were affinity purified using the Pierce Sulfolink Kit (Rockford, IL).

Mutagenesis and transfections

The generation of the two truncation mutants used in this study, N A1–49 and C A253–297, was described previously (27). A 219–225,
Table I.  **PCR primers used in the preparation of CD20 derivatives**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Primer</th>
<th>Sequence of Primer</th>
</tr>
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<tbody>
<tr>
<td>C220A</td>
<td>5'</td>
<td>AAAAGAAGGGCCCTCCAGACCC</td>
</tr>
<tr>
<td></td>
<td>3'</td>
<td>GGGTCTGGAGGCCGTTCTTTT</td>
</tr>
<tr>
<td>CΔ219-225</td>
<td>5'</td>
<td>TGGAAAAAGAAACATAGTTCTCCTGTC</td>
</tr>
<tr>
<td></td>
<td>3'</td>
<td>AAGCTATGTTCTTCCGATCTTCCT</td>
</tr>
<tr>
<td>CΔ226-252</td>
<td>5'</td>
<td>CCAAAACTTCCTTCCCAAACAAAGAAT</td>
</tr>
<tr>
<td></td>
<td>3'</td>
<td>TTGGGAGAAGATTGGTGCTTGAGCA</td>
</tr>
</tbody>
</table>

*All sequences are written in the 5’ to 3’ direction.

CA226–252, and the Cys229/Ala point mutation (C220A) were produced by overlap extension PCR (28) using the internal primer pairs shown in Table I and the CD20 cDNA template provided by Dr. Ivan Stamenkovic (Charlestone, MA). Outside primers used were 5’-ATAATGATTAGGCTT GAGGCTCTTT-3’ (5’ primer that includes a unique EcoRI restriction site at position 451 of the CD20 cDNA) and 5’-AATCAGTTAACAG GAGAGCT-3’ (3’ primer that includes a unique AflII site at position 983). The PCR fragments were digested with EcoRI and AflII, then cloned into pBluescript containing a CD20 cDNA insert from which the EcoRI/AflII fragment had been excised. The sequence of each construct was confirmed before subcloning into the BCGM/Sneo mammalian expression vector (29). Transfection into Molt-4 T cells and selection of geneticin-resistant clones, were performed as previously described (27).

**Immunofluorescence**

Cells were suspended and incubated in RPMI/5% FBS with anti-CD20 or control Abs, washed once, and resuspended in 100 µl (1/100) dilution of either FITC-conjugated rabbit anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) or FITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) or FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) or FITC-conjugated goat anti-rabbit IgG (Tanabe, Beverly, MA) for intracellular staining, Raji B cells (2 × 10^6/sample) were permeabilized with 0.05% saponin in RPMI/5% FBS for 30 min on ice. Subsequent Ab incubation and wash steps were performed as described above, except in the presence of 0.01% saponin. For the peptide inhibition studies, anti-CD20N and anti-CD20C Abs were preincubated with the immunizing peptides CD20N-P and CD20C-P, respectively, for 30 min on ice before incubation. Immunofluorescence studies were performed as previously described (27).

**Trypsin and proteinase K digests**

Raji cells (2 × 10^6/sample) were washed and resuspended in 50 mM Tris-HCl (pH 7.5), then incubated alone or with trypsin (0.2 µg/µl) for 15 min on ice. For proteinase K digests, Raji cells were washed and resuspended in PBS, then incubated alone or with 12.5 µg/ml proteinase K for 15 min on ice. Protease inhibitors (4 mM Pefabloc (Boehringer Mannheim, Laval, Quebec), 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM PMSF) were added to halt digestion. Supernatants were aspirated. Cell pellets were lysed directly in 2× sample buffer, heated to 100°C for 5 min, and loaded on 12.5% polyacrylamide gels (4 × 10^5 cell equivalents/lane).

**Cell stimulation and sample preparation**

Cells (5 × 10^6/sample) were washed in PBS, not treated or treated with 5 µg of 2H7 mAb at 37°C for 15 min, then pelleted and lysed in 20 mM Tris, pH 7.5, containing 0.5% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 5 mM EDTA, and 1 mM PMSF. After 15 min on ice, samples were centrifuged at 14,000 × g for 15 min at 4°C to pellet the insoluble material. Lysates were transferred to clean tubes and mixed with 2× SDS sample buffer. The insoluble pellets were washed four times with lysis buffer and then solubilized in 2× SDS sample buffer. Samples were heated to 100°C for 5 min before separation on 10% polyacrylamide gels (1–3 × 10^5 cell equivalents of lysates/lane; 5–10^5 cell equivalents of pellets/lane). For immunoprecipitation, lysates from 10^7 cells were transferred to clean tubes, incubated overnight at 4°C with either normal rabbit serum or anti-CD20N antiserum (2 µl), then mixed with 20 µl of protein A-Sepharose (Repligen, Cambridge, MA) for 2 h. The Sepharose beads were washed three times with lysis buffer and once with PBS, and then mixed with 2× SDS sample buffer. Prestained m.w. markers (Life Technologies or New England Biolabs (Beverly, MA)) were run on each gel, and proteins were transferred to Immobilon P (Millipore, Bedford, MA) membranes for immunoblotting.

**Immunoblots**

Membranes were blocked overnight with 5% BSA, then incubated for 3 h with anti-CD20N or anti-CD20C Abs diluted in 1% BSA. After washing the membranes, bound Abs were detected with protein A-horseradish peroxidase (Bio-Rad, Richmond, CA). Proteins were visualized using chemiluminescence (Pierce) recorded on Kodak X-OMAT film (Eastman Kodak, Rochester, NY) for the peptide inhibition experiments, anti-CD20N and anti-CD20C Abs were incubated alone or with either CD20N-P or CD20C-P for 30 min on ice before immunoblotting.

**Results and Discussion**

**Specificity of CD20N and CD20C Abs**

To determine the membrane orientation of CD20, Abs against known epitopes were generated in rabbits by immunization with OVA-conjugated CD20 N and C region peptides. The resulting antisera, anti-CD20N and anti-CD20C, were affinity purified and tested for specificity before use in membrane orientation studies (Fig. 1). Specificity was confirmed in three ways. First, both antisera detected proteins of 33 to 35 kDa in immunoblots of whole cell lysates derived from Raji B cells (Fig. 1A). CD20 migrates on SDS-PAGE as a single band, a doublet, or a triplet depending upon the quantity of protein present and the resolving power of the gel. The differently migrating species are the result of differential serine/threonine phosphorylation (30). Preincubation of the Abs with the immunizing peptides inhibited binding, while incubation with irrelevant peptides did not (Fig. 1A). Second, immunoprecipitation with anti-CD20N and detection by immunoblotting with anti-CD20C demonstrated that both Abs recognized epitopes on the same protein (Fig. 1B). Third, both Abs specifically recognized CD20 ectopically expressed by transfection in the Molt-4 T cell line (Fig. 1C).

**Confirmation of the membrane orientation of CD20**

Localization of the N- and C-terminal regions of CD20 was assessed by indirect immunofluorescence using intact and membrane-permeabilized Raji B cells. CD20N and CD20C Abs did not recognize CD20 on intact cells (Fig. 2). However, after permeabilization there was a specific increase in intracellular staining by both anti-CD20N and anti-CD20C Abs that was prevented by preincubation of the Abs with immunizing peptide. Effective permeabilization of the cells was confirmed by the detection of the cytoplasmic Src family tyrosine kinase Lyn only after the permeabilization procedure (data not shown). Since the epitopes recognized by anti-CD20N and anti-CD20C were accessible only after permeabilization, these data confirm the intracellular location of both N- and C-terminal regions of CD20.

The intracellular location of both termini indicates that CD20 must assume one of three possible topologies relative to the plasma membrane (see Fig. 8A). Protease digestion of intact cells was performed to evaluate the number and the locations of extracellular...
loops. Complete digestion of extracellular regions would be expected to result in either 8- or 15-kDa N-terminal fragments depending on whether the polypeptide is exposed at the cell surface on the carboxyl side of TM1. For these studies we used both trypsin, which cuts specifically on the carboxylic acid side of arginine or lysine, and the nonspecific protease proteinase K to treat intact Raji B cells as described in Materials and Methods. There is no trypsin digest site present in the putative loop between TM1 and TM2, and four sites in the hydrophilic region between TM3 and TM4. Therefore, the presence of an extracellular TM1-TM2 loop (Fig. 8 A, ii) would be expected to generate approximately 15- and 8-kDa N-terminal fragments by trypsin and proteinase K, respectively. Further, there are four trypsin sites in the short hydrophilic stretch between TM2 and TM3. These sites would only be exposed in the two transmembrane/one extracellular loop model (Fig. 8 A, i) and would also generate a significantly larger trypsin fragment compared with proteinase K. The only topology that would generate 15-kDa fragments after digestion with either trypsin or proteinase K is shown in Figure 8 A, iii.

The size of the smallest N-terminal fragment generated by digestion of intact cells with either trypsin or proteinase K was slightly >15 kDa (Fig. 3), indicating that the protein does not exit to the cell surface between TM1 and TM3, and that there is a single extracellular loop between TM3 and TM4 (Fig. 8 A, iii). Recognition of more than one band in both the trypsin- and proteinase K-digested samples by CD20N Ab is probably due to serine/threonine phosphorylation as seen in the intact protein. Incomplete digestion by trypsin may account for the third and largest N-terminal fragment detected. Fragments obtained after proteinase K digestion were slightly smaller than that obtained after trypsin digestion, as expected due to clipping of a few extra residues between the most N-terminal trypsin cut site and the outer leaflet of the plasma membrane.

The size of the C-terminal fragments (≈21 kDa) resulting from digestion by either trypsin or proteinase K significantly exceeded the expected size (14 kDa), but is too large to be accounted for by incomplete digestion of the extracellular loop. The discrepancy in size may be attributable to post-translational modifications in the C-terminal region, such as phosphorylation and/or acylation (dis-
cussed further below), or to SDS-resistant protein-protein interactions occurring after digestion.

Since the epitopes recognized by anti-CD20N and anti-CD20C were not destroyed by protease digestion, these results, together with those in Figure 2, confirm their intracellular location. Data from extracellular protease digestion analyses support a four-TM domain topology with a single extracellular loop between TM3 and TM4, in agreement with the hydropathy prediction.

Generation of CD20 deletion mutants

We recently reported that CD20 redistributes from the soluble to the insoluble fraction of Triton X-100 cell lysates when B cells are exposed to Abs directed against extracellular epitopes of CD20 before lysis. The speed with which CD20 can be induced to translocate (6) indicates that the mechanism does not require de novo transcription or translation and suggests a conformational and/or post-translational change in the CD20 molecule. To identify regions that might be involved in the process of CD20 redistribution, we generated a series of cytoplasmic domain deletion mutants and tested them for their ability to translocate to the Triton-insoluble fraction following CD20 mAb engagement. Generation of a deletion construct that lacked the entire N-terminal cytoplasmic region (NΔ1–49) was described previously (27) and was detected in immunoblots using the polyclonal anti-CD20C Ab. As reported previously (27), deletion of the C-terminal region from either residue 215 or 222 to the end of the molecule at position 297 resulted in no or poor expression, respectively. However, internal deletions were expressed well (Fig. 4), and the C-terminal region was therefore analyzed using three constructs, CΔ219–225, CΔ226–252, and CΔ253–297, that were detected in immunoblots with the anti-CD20N Ab. As reported previously (27), deletion of the C-terminal region from either residue 215 or 222 to the end of the molecule at position 297 resulted in no or poor expression, respectively. However, internal deletions were expressed well (Fig. 4), and the C-terminal region was therefore analyzed using three constructs, CΔ219–225, CΔ226–252, and CΔ253–297, that were detected in immunoblots with the anti-CD20N Ab. As reported previously (27), deletion of the C-terminal region from either residue 215 or 222 to the end of the molecule at position 297 resulted in no or poor expression, respectively. However, internal deletions were expressed well (Fig. 4), and the C-terminal region was therefore analyzed using three constructs, CΔ219–225, CΔ226–252, and CΔ253–297, that were detected in immunoblots with the anti-CD20N Ab. All constructs were stably expressed in Molt-4 T cells, and expression levels were monitored by indirect immunofluorescence using the 2H7 mAb against an extracellular epitope (Fig. 4). Clones expressing either high or lower levels of wild-type CD20, as shown in Figure 4, were selected and used in different experi-

Effects of cytoplasmic deletions on CD20 redistribution

Cells were incubated with or without 2H7 anti-CD20 mAb and lysed in Triton X-100-containing buffer, and both soluble and insoluble fractions were collected as described in Materials and Methods. The presence of CD20 in the soluble lysates or insoluble pellets was detected by immunoblot after SDS-PAGE, and the amounts were estimated by densitometry analysis of quadruplicate samples. In untreated cells, CD20 was always found entirely in the Triton-soluble fractions. As reported previously for CD20 on B cells, Ab-mediated ligation of wild-type CD20 in the transfected Molt-4 T cells induced virtually all CD20 protein to translocate to the insoluble compartment. This was observed both by the disappearance of CD20 from the soluble lysates and by its appearance in the insoluble compartment (Fig. 5). In contrast, a substantial amount of CD20 remained in the soluble compartment after Ab treatment when seven amino acids in the membrane-proximal region of the cytoplasmic C-terminal domain (CΔ219–225) were deleted (Fig. 5). The inhibition was not complete, however, as most clearly observed by the detection of some CD20 in the insoluble pellets.
Densitometry analysis of quadruplicate samples estimated the degree of translocation to be about 25% compared with almost 100% for wild-type CD20. Deletion of an adjacent stretch of 26 residues (CD226–252) inhibited translocation also, albeit to a lesser extent (~75% translocated), whereas deletion of the C-terminal 45 residues (CD253–297) had no discernible effect on Ab-induced translocation. The CD20 mutant lacking the N-terminal region (NΔ1–49) was detected using the anti-CD20C polyclonal antiserum. This antiserum exhibited some background reactivity; nevertheless, it was clear in multiple experiments that the N-terminal deletion minimally reduced Ab-induced CD20 translocation by about 10% (Fig. 6).

Potential palmitoylation of Cys220 is not required for CD20 redistribution

The sequence 219 to 225 includes a cysteine residue at position 220 that is conserved in the other two members of the CD20 family of related proteins, i.e., FcERIβ and HTm4 (31, 32). The proximity of Cys220 to the inner leaflet of the plasma membrane makes it a potential site of reversible lipid modification by palmitoyl transferase. Palmitoylation is required for the caveolar localization of the Src family tyrosine kinase Hck and the endothelial nitric oxide synthase (33, 34), and is found on a number of other proteins in these microdomains, including several of the Gα signaling proteins and caveolin, a structural protein of caveolae. To determine whether a requirement for palmitoylation of Cys220 accounted for the inhibitory effect of the 219 to 225 deletion, alanine was substituted for cysteine at this position. Expression of C220A on transfected Molt-4 cells, as measured by indirect immunofluorescence, is shown in Figure 4. Mutation of Cys220 to alanine did not prevent or reduce Ab-induced translocation of CD20 to the insoluble pellet (Fig. 7), eliminating palmitoylation, or other potential modifications, of Cys220 as a mediator of CD20 redistribution. A second cytoplasmic cysteine is present in the loop between the second and third transmembrane regions; however, this cysteine is not conserved in FcERIβ, HTm4, or murine CD20, making it an unlikely candidate for an essential function. Since deletion of the internal loop prevented high level expression of CD20 (27), we could not detect the mutated protein in immunoblots and were unable to assess the impact of this deletion on CD20 translocation (data not shown).

Dynamic targeting of CD20 to detergent-insoluble domains is shown here to be dependent on a membrane-proximal sequence in the C-terminal cytoplasmic region (Fig. 8B). Although this region contains a potential palmitoylation site, this site was not required for the translocation of CD20. A similar observation was reported for caveolin; although palmitoylated, this modification is not required for caveolar localization of caveolin (35). Since multiple potential phosphorylation sites exist in both 219 to 225 and 226 to 252 sequences, it is possible that phosphorylation of CD20 is required for its translocation. CD20 is known to be phosphorylated (36, 37), and the level of phosphorylation is increased upon activation with phorbol esters (PMA) or Abs directed against surface IgM (37–40). The PMA-induced increase in phosphorylation of CD20 suggests that PKC may phosphorylate CD20 in vivo, as it does in vitro (39). Interestingly, however, we found that pretreatment of B cells...
CD20, when aggregated, redistributes to detergent-insoluble microdomains in B cells upon Ab engagement. It is likely that the redistribution of CD20 to insoluble membrane microdomains is a prerequisite event to the initiation of signaling and/or calcium channel formation. The identification of a cytoplasmic sequence that prevents CD20 redistribution is expected to lead to a full understanding of the underlying mechanism and perhaps to new insights into B cell activation.

Acknowledgments

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References


