The CD20 Calcium Channel Is Localized to Microvilli and Constitutively Associated with Membrane Rafts

ANTIBODY BINDING INCREASES THE AFFINITY OF THE ASSOCIATION THROUGH AN EPITOPE-DEPENDENT CROSS-LINKING-INDEPENDENT MECHANISM*

Haidong Li‡§, Linda M. Ayer‡§, Maria J. Polyak‡, Cathlin M. Mutch‡, Ryan J. Petrie‡, Laura Gauthier‡, Neda Shariat‡, Michael J. Hendzel, Andrew R. Shaw‡, Kamala D. Patel‡, and Julie P. Deans‡**

From the ‡Department of Biochemistry and Molecular Biology, and the §Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, and the ¶Department of Oncology, University of Alberta, Edmonton, Alberta T6G 1Z2, Canada

CD20 is a B cell-specific tetraspan protein that assembles into oligomeric complexes and forms or regulates a store-operated calcium entry channel that is responsive to B cell receptor (BCR) signaling (1). CD20 is also an effective target for in vitro depletion of malignant or autoimmune B cells using monoclonal antibodies (mAbs), which can activate apoptotic signaling pathways and mediate complement-mediated cytotoxicity potentially through mechanisms involving cholesterol- and sphingolipid-rich membrane microdomains known as lipid rafts (2–4). Rafts are thought to function in part as platforms for signaling from those receptors with properties that allow their access to the tightly packed lipid raft environment, which otherwise excludes most membrane proteins (5–7). The operational criteria for assigning raft association of a protein are insolubility in nonionic detergents and buoyancy on density gradients. The detergent best characterized for raft isolation is Triton X-100, and we showed previously that antibodies induce translocation of CD20 from the soluble fraction of Triton X-100 cell lysates into the buoyant insoluble fraction, consistent with its induced association with rafts (8). However, raft association of some proteins can only be demonstrated using very low concentrations of Triton X-100 or other nonionic detergents (9–11), and we recently found that unligated CD20, although soluble in 1% Triton X-100, was insoluble in 1% Brij 58 (1). Brij 58-insoluble CD20 localized to cholesterol-dependent, buoyant fractions on sucrose density gradients. Importantly, deletion of a short membrane-proximal cytoplasmic sequence, previously shown to be essential for efficient translocation into Triton-resistant rafts (12), also prevented the constitutive association of CD20 with Brij 58 buoyant fractions. CD20 thus appears to be an example of a raft-associated protein that is Triton-soluble. What, then, is the meaning of antibody-induced translocation into Triton-resistant rafts? Here, we first extend our observations on the constitutive nature of CD20-raft association and then explore the effects of antibody ligation on cell surface distribution and mechanisms of antibody-induced Triton-insolubility of CD20.

Our results show that antibody binding does not induce a detectable alteration in cell surface distribution of CD20, which in the absence of antibody binding is already distributed unevenly on the B cell surface. Transmission electron microscopy (EM) demonstrated that CD20 was localized to membrane protrusions, or microvilli. Anti-CD20 mAbs differ dramatically in their ability to induce Triton insolubility of CD20, and we show clear cells; GFP, green fluorescence protein; A488, Alexa 488; CTB, cholera toxin subunit B; PFA, paraformaldehyde; GM1, Galα1,3GalNAcβ1,4NeuAcα2,3Galβ1,4Glcβ1,1-ceramide; MBC, methyl-β-cyclodextrin; NaN₃, sodium azide; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid.
here that these differences cannot be attributed to the antibody isotype or to the amount of antibody bound per cell but are epitope-dependent. Translocation into Triton-resistant rafts is shown to be independent of the availability of ATP, kinase activity, actin polymerization, and the cross-linking effects of antibodies. Together, these data are consistent with the interpretation that CD20 is constitutively associated with lipid rafts on microvesicles and that antibody engagement increases the affinity of the association through an intrinsic mechanism, such as a conformational change in the CD20 protein.

**EXPERIMENTAL PROCEDURES**

**Cells—**Ramos and BJAB B cells were maintained in culture in RPMI 1640 plus 7.5% fetal bovine serum. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using lymphocyte separation medium (ICN Biomedicals, Aurora, OH). Stable transfectants of BJAB cells expressing green fluorescence protein (GFP)-CD20 were generated by electroporation at 250 V and 500 microfarads (Gene Pulser II; Bio-Rad) with 30 μg of DNA. GFP was fused to the amino terminus of CD20 by inserting human CD20 cDNA into the cloning site of the pEGFP expression vector (Clontech) using XhoI/SacII. CD20-positive cells were sorted by flow cytometry (FACStar cytometer; BD Biosciences) and maintained with Geneticin (Invitrogen) at 1 mg/ml.

**Antibodies and Reagents—**Monoclonal antibodies 2H7 (IgG2b anti-CD20) and 9.4 (IgG2b anti-CD45) were provided by Dr. J. Ledbetter (Seattle, WA), and the isotype switch variants of NK1.2.B20 were provided by Dr. Anne-Marie Hekman (Netherlands Cancer Institute, Amsterdam) (13). B1 (IgG2a anti-CD20) was purchased from Coulter (Hialeah, FL). Anti-CD59 antibody (MEM43/5) was purchased from Sanbio (Uden, The Netherlands). 2H7 and B1 were conjugated to Alexa 488 (A488) using the Alexa Fluor 488 protein labeling kit (Molecular Probes, Inc., Eugene, OR). Goat anti-rabbit IgG conjugated to Cy3 was purchased from Jackson ImmunoResearch. Cholera toxin subunit B (CTB) and rabbit anti-CTB were purchased from Sigma. For immunoblotting, anti-Ga, antibody was purchased from Oncogene (Boston, MA), anti-Lyn from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-actin from Roche Applied Science, and anti-CD45 antibody from Transduction Laboratories (Lexington, KY). Rabbit antiserum directed against a CD20 cytoplasmic region peptide was generated as described (14). Horseradish peroxidase conjugates of Protein A, rabbit anti-mouse IgG, and CTB were purchased from Bio-Rad, Southern Biotechnology Associates (Birmingham, AL), and Sigma, respectively.

Fab fragments of 2H7 were produced using the ImmunoPure Fab preparation kit (Pierce). Purity was assessed by SDS-PAGE separation of titrated 2H7 mAb, both intact and digested, followed by Western blot using biotin-conjugated anti-mouse IgG2b detected with avidin-horse-radish peroxidase (both reagents from Southern Biotechnology Associates (Birmingham, AL), and Sigma, respectively.

FIG. 1. CD20 localizes to the buoyant fractions of most detergent lysates. Ramos B cells were lysed in the detergents indicated to the right. After sucrose density centrifugation, equal cell equivalents of fractions 1–8 and the insoluble pellet (fraction P) were analyzed by immunoblot as indicated to the left. Results are representative of at least three experiments.
imaging system. Quantity One software was used to quantitate the signal.

**Flow Cytometry**—Cells were incubated with anti-CD20 mAb, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. The data were acquired using a Becton Dickinson FACScan (BD Biosciences) and analyzed using the FlowJo program (Tree Star, Inc., San Carlos, CA).

**Immunofluorescence Imaging**—Cells were fixed in 1% PFA at room temperature for 5 min and then incubated with antibody as indicated or incubated with antibody prior to fixation. Fixed and unfixed PBMC (10⁶) were incubated with 1 μg of 2H7-A488 in 100 μl of PBS at 37°C for 15 min. Fixed Ramos B cells were incubated with B1- or 2H7-A488 (1 μg/10⁶ cells) for 30 min at 37°C; unfixed Ramos cells were incubated with the same antibodies for 10 min at 37°C, washed, and fixed. For colocalization studies, 10⁶ BJAB (GFP-CD20) cells were fixed in 1% PFA; incubated with anti-CD59, anti-CD20, or anti-CD45; washed; and further incubated with anti-mouse IgG-Cy3. Fluorescence imaging was done with a Leica DM RXA microscope attached to a 14-bit cooled CCD camera (Princeton Instruments, Monmouth Junction, NJ). Digital deconvolution was performed using the MicroTome software (VayTek, Fairfield, IA). In some experiments, imaging of PBMC was with DeltaVision Image Restoration Microscopy System (Applied Precision, Issaquah, WA).

**Electron Microscopy**—For transmission EM, Ramos cells (1 × 10⁷) were incubated with mouse IgG anti-CD20 (15 μg in 500 μl of PBS), washed in PBS, and then incubated in colloidal gold (6 nm)-conjugated goat anti-mouse IgG (Electron Microscopy Sciences). After washing, the cells were fixed in cold 2.5% glutaraldehyde in PBS with rotation at 4°C for 1 h. After washing three times (20 min each) with the same buffer, the cells were pelleted and embedded in 3% agar. The agarized pellet was cut into 1-mm slices and immersed in 1% osmium tetroxide in PBS for 1 h at room temperature. The pellets were rinsed in distilled water, dehydrated in graded ethanol series, and embedded in Epon 812. Thin sections were cut with a diamond knife, mounted on naked copper grids, and viewed with a Hitachi (Schaumburg, IL) H-7000 transmission electron microscope.

For scanning EM, Ramos cells (1 × 10⁷) were fixed in cold 1% glutaraldehyde/PBS with rotation at 4°C for 1 h, washed three times in PBS, and dehydrated in graded ethanol series. The cell pellet was resuspended in 10 μl of 100% ethanol and transferred to a poly-L-lysine-coated coverslip for critical point drying. Coverslips were then sputter-
coated with gold palladium, dried, and mounted on the sample stage of a Philips XL30 scanning electron microscope (FEI Co., Hillsboro, OR).

RESULTS

CD20 Is Constitutively Associated with Membrane Rafts—To test whether the use of detergents other than Triton X-100 might reveal a significant presence of CD20 in rafts in the absence of antibody engagement, Ramos B cells were lysed in the series of detergents indicated in Fig. 1. After sucrose density gradient centrifugation, fractions were tested by immunoblot for the presence of CD20. As controls, the gradient fractions were also blotted for actin, raft markers GM1 and G/H9251, and nonraft marker CD45. CD20 was solubilized by Triton X-100 at 1%, as expected. However, in 0.05% Triton and in all other detergents except Brij 96, 30–80% of CD20 redistributed to the buoyant raft fractions 3–5. Glycosphinglipid GM1 localized to the raft fractions in all detergents. The acylated hetero-

three-dimensional reconstructions of all deconvolved sections through the cells, as indicated. B, Ramos cells were fixed and then labeled with A488-conjugated Fab fragments of 2H7 or A488–2H7 mAb as indicated. C, Ramos cells were fixed with 1% PFA, stained with B1-A488 or 2H7-A488 (left panels), or incubated at 37 °C with the same antibodies and then fixed (right panels). D, BJAB cells stably transfected with GFP-CD20 were lysed in 1% Brij 58 and fractionated by sucrose density gradient centrifugation. Fractions were probed by anti-CD20 immunoblot. E, GFP-CD20-transfected BJAB cells were incubated at 37 °C with antibody or with 2H7 or B1 mAbs as indicated. F, GFP-CD20 BJAB cells were incubated with anti-CD59 or anti-CD45 antibodies, and bound antibodies were visualized with Cy3-conjugated secondary antibody. Images separately acquired using GFP (λex/λem = 470/525) and Cy3 (λex/λem = 535/610) filter combinations were deconvolved and merged. Yellow indicates areas of overlapping distribution.
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Fig. 5. Antibody-induced insolubility of CD20 in 1% Triton. A, untreated, MBC, or cholesterol/MBC-treated Ramos cells were incubated with isotype control or 2H7 mAb before lysis in 1% Triton X-100, fractionated on sucrose density gradients, and immunoblotted for CD20 or Lyn. B, Ramos cells were pretreated with MBC for the times indicated, and then 2H7 anti-CD20 was added for an additional 15 min before lysis in 1% Triton X-100. The soluble and insoluble fractions were collected and probed by immunoblot for CD20. Results are representative of three experiments in A and two experiments in B. For these and similar experiments shown in later figures, equal loading was demonstrated by actin blot or Coomassie Blue staining, but it is not shown for clarity of presentation.

CD20 Is Localized to Microvilli—In three-dimensional reconstructions of deconvolved optical sections such as those shown in Fig. 3A, it appeared that CD20 staining was clustered on...
membrane protrusions. To examine this at higher resolution, Ramos B cells were labeled with CD20 mAb and gold-conjugated anti-mouse IgG and prepared for transmission EM. A grid was placed over each of 39 images derived from different cells in two experiments, and gold particles associated with membrane protrusions or with the flat continuous part of the plasma membrane were enumerated. Among a total of 656 particles counted, 591 (90%) were associated with membrane protrusions. A representative image is shown in Fig. 4; low magnification of transmission and scanning EM images of Ramos B cells in the lower panels of Fig. 4 illustrate the gross plasma membrane architecture of these cells, which is similar to that reported previously for primary B cells (16–19). No gold particles were observed in isotype control samples (data not shown).

**Antibody-induced Insolubility in 1% Triton**—Although unligated CD20 is soluble in 1% Triton, antibody binding can induce almost complete insolubility of CD20 in this detergent (8) (see Fig. 5A). Antibody-ligated Triton-insoluble CD20 was found in the low density region of sucrose gradients and not in the high density pellet (Fig. 5A, fraction P). The buoyancy of ligated Triton-insoluble CD20 was sensitive to cholesterol depletion and restored by subsequent incubation with cholesterol-loaded MBC (Fig. 5A). The distribution of the Src-family kinase Lyn on these gradients is shown for comparison. In light of the constitutive CD20-raft association demonstrated earlier, these data suggest that induced Triton insolubility reflects increased strength of the association with rafts. We then investigated the mechanism underlying this effect using a rapid and sensitive procedure, described previously (12), in which the loss of CD20 from the soluble fraction of Triton lysates caused by antibody binding is monitored as well as the corresponding gain of CD20 in the insoluble material obtained after microcentrifugation. To confirm the cholesterol dependence of CD20 insolubility using this procedure, Ramos B cells were pretreated with MBC before the addition of 2H7 anti-CD20 mAb.
Both soluble and insoluble fractions were collected and probed for the presence of CD20 (Fig. 5B). As expected, antibody-induced loss of CD20 from the soluble fractions (lane 5) was prevented by preincubation of the cells with MBC (lanes 6–8). The pellet fractions showed the reverse results (i.e. 2H7-induced CD20 Triton-insolubility was prevented by MBC pretreatment).

Antibody-induced association of CD20 with 1% Triton-resistant rafts could potentially be mediated by the actin cytoskeleton, by signaling events or post-translational modifications, by clustering on a scale that is not obvious at the level of light microscopy, or by a CD20-intrinsic mechanism such as a change in conformation. Cytoskeleton-associated proteins are among the most abundant proteins in membrane rafts (20). The membrane skeleton has also been suggested to regulate the size of lipid rafts (21). As shown in Fig. 6A, pretreating Ramos cells with 1/10M cytochalasin D for up to 16 h did not affect CD20 Triton insolubility induced by 2H7, as compared with control cells. Similar results were obtained with 10 μM cytochalasin D and with cytochalasin E (data not shown). Cytochalasin D at 1 μM effectively disrupted the actin cytoskeleton as assessed by inhibition of antibody-induced BCR capping and cellular aggregation (data not shown), which require an intact cytoskeleton (22, 23).

To test whether antibody-induced Triton resistance of CD20-raft association is an active process that requires energy, we used sodium azide (NaN₃) and deoxyglucose to inhibit ATP generation before antibody addition. As shown in Fig. 6B, induced CD20 Triton insolubility occurred even at the highest concentrations used. The treatments effectively inhibited ATP generation as shown by the disappearance of the upper band, which represents the more heavily phosphorylated form of CD20 (24), and by inhibition of homotypic aggregation (data not shown).

Phosphorylation can mediate protein-protein and protein-lipid interactions. CD20 itself is phosphorylated at multiple sites by serine/threonine kinases (25) and activates tyrosine kinases after antibody cross-linking (26). Therefore, we tested whether inhibitors of serine/threonine kinases (staurosporine) and Src family kinases (PP1) have any effect on antibody-induced Triton resistance of CD20-raft association. As shown in Fig. 6, C and D, neither inhibitor prevented the induction of CD20 Triton insolubility. Reduced presence of the upper CD20 band after exposure to staurosporine indicates that the treatment was effective (Fig. 6C). The effectiveness of the PP1 treatment was confirmed by its ability to inhibit BCR-stimulated tyrosine kinase-dependent calcium mobilization (data not shown). Together with the results in Fig. 6B, these data indi-
cate that antibody-induced insolubility of CD20 in 1% Triton occurs independently of phosphorylation events.

**Antibody-induced Triton Resistance of CD20-Raft Association Is Epitope-dependent and Cross-linking-independent**— Anti-CD20 mAbs show marked differences in the amount of CD20 Triton resistance they induce (8, 15) (see below). A potential cause of the variability could lie in isotype differences among CD20 mAbs. However, heavy chain isotype switch variants IgG1, IgG2a, and IgG2b of a single antibody specificity NK1-B20 (13) induced equivalent amounts of CD20 Triton insolubility, thus eliminating isotype differences as an underlying cause of the variability observed (fig. 7A).

Recently, we demonstrated that B1 and 2H7 recognize distinct epitopes on CD20 and provided evidence strongly suggesting that the anti-CD20 mAb, Bly1, recognizes the same epitope as B1 (15). AT80 and Rituximab are two of several CD20 mAbs sharing similar fine specificity NK1-B20 (13) induced equivalent amounts of CD20 Triton insolubility, thus eliminating isotype differences as an underlying cause of the variability observed (fig. 7A).

Our conclusion that CD20 is constitutively localized to lipid rafts is supported by evidence from both biochemical and cellular studies. Unligated CD20 localized to low density detergent-resistant membranes isolated using Brij 35, Brij 58, Thesit, CHAPS, or a low concentration of Triton X-100. When isolating rafts using detergents that may be less stringent than 1% Triton, a significant concern is that nonraft membrane regions are inadequately solubilized. We controlled for this possibility using CD45, which is abundantly expressed on B cells, as it is on all leukocytes, and is not generally found in lipid rafts. The low density membranes isolated in our experiments did not include CD45. Localization of CD20 to rafts was cholesterol-dependent as expected and, as shown previously, prevented by deletion of 7 residues in a membrane proximal cytoplasmic region of CD20 (1, 12). At the cell surface, unligated CD20 co-localized with rafts, as indicated by overlapping distribution of GFP-CD20 with a Cy3-labeled glycosylphosphatidylinositol-linked protein, CD59. Co-localization was not complete, however, suggesting that CD20 and CD59 may also occupy distinct rafts. Heterogeneity among rafts in B cells has been documented previously (14). In contrast, no co-localization between CD20 and CD59 was evident.

**DISCUSSION**

We compared the structures of the detergents used in this study with their ability to retain CD20, GM1, and G8/H9251 in low-density detergent-insoluble fraction (Fig. 3C), although not to the same extent as intact antibody. The reduced level of CD20 Triton insolubility induced by monovalent Fab is consistent with reduced binding to CD20, relative to whole 2H7 mAb, as assessed by flow cytometry (Fig. 7E). Cross-linking is therefore neither sufficient (since B1 and Bly1 are ineffective) nor required to induce Triton resistance of CD20-raft association.
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dept. 100 raft fractions. Among the nonionic detergents, only Triton X-100 has a bulky p-isooctyl phenyl group (Table I), perhaps accounting for the ability of high concentrations of Triton to solubilize some raft proteins like CD20. Brij 96 behaved most similarly to Triton. Although Brij 96 has no phenyl group, there is a double bond in its oleyl chain not found in Brij 35, Brij 58, or Thesit. This and/or other distinctive features of Brij 96, might confer, like Triton, a greater ability to destabilize protein-raft associations. In contrast, the interactions of glycosphingolipid GM1 with other raft lipids were resistant to both Triton and Brij 96. Following a similar trend, the lipid-raft interactions of Gαq were resistant to Triton X-100 and to some extent also to Brij 96. This analysis suggests that CD20 is unlikely to associate with rafts via modification with long chain fatty acids. Although there are potential sites for palmitoylation at Cys110 and Cys220, mutation of either or both sites did not prevent CD20 raft association (12) (data not shown). CHAPS is considered a stringent detergent in isolating raft-associated proteins (11), yet it retained most of CD20 in rafts. CHAPS is a steroid-based zwitterionic detergent frequently used to extract transmembrane proteins in their functional configuration, presumably by preserving protein-lipid interactions while disrupting protein-protein interactions. Thus, the resistance of CD20 to CHAPS may indicate that its constitutive raft localization is mediated by interactions with lipids rather than proteins. Consistent with this notion, the conformation of CD20 was recently shown to be sensitive to the level of membrane cholesterol, suggesting a potentially direct CD20-cholesterol interaction (27).

The restricted distribution of CD20 on microvilli is consistent with its co-localization with the BCR (14), which is also found on microvilli, presumably to facilitate interaction with antigen (28, 29). In T cells, L-selectin also has microvillar localization and associates with rafts (30, 31). Interestingly, the pentaspan microvilli-localized protein prominin is also associated with cholesterol-dependent lipid rafts that are soluble in Triton X-100 but insoluble in other nonionic detergents (32). In cells at the intestinal brush border, several transmembrane digestive enzymes and galectin 4 localize to Triton-sensitive rafts on microvilli (33, 34). Thus, microvillar rafts may be a special subset with characteristics that attract proteins that associate with intracellularly bulkier affinity.

Antibody-induced effects on CD20-raft association are particularly important in the context of the therapeutic use of anti-CD20 antibodies (2). The ability of anti-CD20 antibodies to fix complement has been correlated with their ability to induce Triton-resistant raft association (4), and the activation of Src family kinases by CD20 cross-linking, which can lead to apoptotic cell death, is likely also a raft-dependent effect (2). Antibody cross-linking can stabilize weak protein-raft interactions or cause partitioning of proteins into rafts. In the case of CD20, cross-linking was shown here to be neither sufficient nor required to induce a transition to a high affinity raft-associated state resistant to Triton extraction. The antibody effect was epitope-dependent and inducible by highly purified Fab fragments. Together with our data excluding involvement of energy-dependent processes, these observations point to an intrinsic mechanism underlying a transition to high affinity raft association.

The epitope-dependent property of CD20 antibody engagement described here raises the question of possible physiological extracellular interactions that could mediate a similar effect. One consequence of such an interaction could be increased sensitivity of the store-operated calcium channel that is formed or regulated by CD20. Calcium influx in BCR-stimulated cells is significantly reduced by cholesterol depletion as well as by down-regulation of CD20, suggesting that the raft environment of CD20 may be important for channel function. Deletion of the sequence in CD20 that controls raft association also abolished calcium entry (1). Thus, increased strength of the association with rafts, mediated by a conformational change induced by an extracellular stimulus, could result in enhanced or prolonged calcium entry. Indeed, anti-CD20 mAbs have been shown to enhance calcium entry, with 1F5 performing better than B1 in this regard (35). The 1F5 mAb was not used in the current study but was shown previously to induce the association of CD20 with Triton-resistant rafts (8), suggesting a correlation between antibody-enhanced calcium entry and high affinity raft association.

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