Rapid Redistribution of CD20 to a Low Density Detergent-insoluble Membrane Compartment*

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CD20 is a B cell integral membrane protein capable of initiating growth-modulating signals in human B lymphocytes upon its engagement with monoclonal anti-CD20 antibodies. In this report, we demonstrate that treatment of B cells with CD20 antibodies induces rapid redistribution of CD20 into a detergent-insoluble membrane compartment. Redistribution is detected as early as 15 s, following antibody addition, and involves up to 95% of CD20 molecules, depending on the antibody used. All of the detergent-insoluble CD20 was found in the low density fractions of sucrose density gradients, indicating that CD20 redistributes to glycolipid-rich membrane domains, analogous to caveolae in some cell types. As CD20 has previously been shown to associate with Src family tyrosine kinases, their co-existence in these compartments suggests a link to the role of CD20 in signal transduction. This study provides insight into the mechanism by which CD20 communicates signals to the cell interior and indicates that the search for membrane-proximal intracellular signaling partners should be directed to the Triton-insoluble fraction.

The plasma membrane provides both a barrier and an interface to the extracellular microenvironment and supports many cell surface proteins responsible for transmitting extracellular signals to the cell interior. Increasing evidence supports the notion that, for some receptors, signal integration involves the nonrandom distribution of signaling proteins into subcompartments in the plasma membrane (1, 2). Local variation in lipid content provides one mechanism for the organization of microdomains in the plasma membrane and permits their biochemical isolation on the basis of detergent insolubility and buoyancy on sucrose density gradients (3). In lymphocytes, it has been established that glycosylphosphatidylinositol (GPI)-linked proteins and Src family kinases are enriched in these detergent-insoluble microdomains (4, 5), but few integral membrane proteins have been localized to these areas (6).

CD20 is a 33–35-kDa nonglycosylated integral membrane protein expressed on all B lymphocytes from the late pre-B cell stage until it is lost just prior to terminal differentiation into plasma cells. The cDNA sequence of CD20 predicts that it spans the plasma membrane four times, with an intracellular location for both amino and carboxyl tails and a short extracellular loop between the third and fourth transmembrane domains (7–9). Monoclonal antibodies (mAb) directed against extracellular epitopes of CD20 can modulate B cell growth and differentiation, indicating the ability of CD20 to transduce extracellular signals (10–15). A signaling role for CD20 is further supported by the activation of tyrosine kinase activity in B cells after their exposure to CD20 mAbs (16). In addition, evidence suggests that CD20 may also be involved in the control of intracellular free calcium concentration by regulating both calcium influx (17–19) and mobilization of calcium from intracellular stores (16).

CD20 is tightly associated with Src family kinases and with an unidentified tyrosine-phosphorylated protein of 75–80 kDa (p75/p80) (20). However, the mechanism underlying the ability of CD20 to transmit signals regulating B cell activation has not been further elucidated. To explore the molecular basis for differing effects of CD20 mAbs on B cell activation, the ability of three of these antibodies to co-precipitate CD20, kinase activity, and tyrosine-phosphorylated proteins was compared. The results of this study uncovered the remarkable ability of CD20 to redistribute to a Triton-insoluble, low density membrane compartment upon binding to anti-CD20 mAb. In many cell types, this compartment has been equated with caveolae, defined morphologically as microinvaginations in the plasma membrane, and more recently, by the presence of caveolin, a resident caveolar protein (21). In lymphocytes, the apparent absence of both caveolin and morphologically defined caveolae leaves their designation provisional in this cell type (22). However, biochemically similar structures, sometimes referred to as “rafts” or DIGS (detergent-insoluble glycolipid-enriched structures) (4, 5), have been isolated from lymphocytes, and we propose that active relocalization of CD20 to these microdomains is a necessary event in the initiation of CD20 signaling. In support of this, an activating anti-CD20 mAb was found to uniquely induce the appearance in the same compartment of a tyrosine-phosphorylated protein of ~50 kDa.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**Raji B cells were maintained by culture in RPMI 1640 medium, 5% fetal bovine serum. The CD20-specific mAbs, 2H7 and 1F5, were provided by Dr. J. Ledbetter (Bristol-Myers Squibb, Seattle, WA). The CD20 mAb B1 was purchased from Coulter Corp. (Hialeah, FL). Rabbit antiserum to CD20 was generated using a peptide corresponding to amino acids 25–41 of human CD20 conjugated to ovalbumin. Anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology Inc.

**Cell Stimulation and Sample Preparation—**Raji cells were warmed to 37 °C before incubation with 2H7, 1F5, B1 or isotype-matched control mAbs (1 µg/ml) cells) and then pelleted and lysed in ice-cold 0.5% Triton X-100 (Pierce) containing 20 mM Tris, pH 7.5, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA.

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§ The abbreviations used are: GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid.
In time course experiments requiring very short exposure to antibody, cells were not pelleted but were lysed in 2 × 10^5 Triton X-100 detergent buffer. After 15 min on ice, lysates were centrifuged at 14,000 × g for 15 min at 4 °C to separate out the insoluble material. The insoluble pellets were washed 4 times in lysis buffer before addition of 2 × SDS sample buffer and then subjected to 2 cycles of heating to 100 °C for 5 min, vortexing, and freezing. For immunoprecipitation, post-nuclear lysates were transferred to clean tubes and mixed with 25 μl of protein A-Sepharose (Repligen Corp., Cambridge, MA) for 2 h. For in vitro phosphorylation, immune complexes precipitated from cell lysates with protein A-Sepharose beads were washed three times in lysis buffer, twice in kinase buffer (20 mM Pipes, pH 7.2, 10 mM MgCl2, and 5 mM MgCl2), and resuspended in 25 μl of kinase buffer containing 5 μCi of [γ-32P]ATP (specific activity, 3000 Ci/mmol, Amersham Corp.). Reactions were allowed to proceed at room temperature for 15 min and then were stopped by the addition of 3 μl of 0.5 mM EDTA. Samples were washed with lysis buffer, heated to 100 °C for 5 min in 2 × SDS sample buffer, run on 10% polyacrylamide gels, and transferred to Immobilon P (Millipore). Bands were visualized by autoradiography using Kodak X-OMAT film.

**Immunoblotting**—Membranes were blocked in 5% bovine serum albumin, incubated with 1/2000 dilution of CD20 antiseraum or 4G10 mAb, and washed, and then bound antibody was detected using protein A-horseradish peroxidase (Bio-Rad) or anti-mouse IgG-horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) and developed by chemiluminescence (Pierce). Pre-stained molecular weight markers (Bio-Rad or NEB) were run on each gel. Bands were visualized using Kodak X-OMAT film (Eastman Kodak Co.)

**Immunofluorescence**—Cells (2 × 10^6) were suspended and incubated in 100 μl of RPMI 1640 medium, 10% fetal bovine serum for 15 min at 37 °C with 2H7, 1F5, B1, or isotype-matched control mAb, washed once, and resuspended for 15 min with 100 μl of 1/100 dilution of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Southern Bio-technology Associates, Inc.). Experiments as represented in Fig. 5 were performed with modifications to incubation times as described in the figure legend. After washing, cells were resuspended in phosphate-buffered saline/0.01% azide and analyzed on a FACScan cytomter (Becton Dickinson).

**Sucrose Density Gradient Analysis**—Raji cells (1 × 10^7 per gradient) were lysed in 1% Triton X-100 in MES-buffered saline (MBS; 25 mM MES, pH 6.5, 150 mM NaCl, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), mixed with sucrose to a final concentration of 40%, overlayered with a 5–30% sucrose density gradient as described (3, 23), and then centrifuged at 37,000 rpm for 16 h. Fractions corresponding to 12–20% sucrose on the gradient were collected, diluted in MBS such that the final concentration of sucrose was less than 5%, and pelleted by centrifugation at 37,000 rpm for 1 h, and the Triton X-100 insoluble pellets were then solubilized in SDS sample buffer.

**RESULTS**

Co-precipitation of p75/80 by Different CD20 mAb Is Inversely Proportional to the Amount of CD20 Precipitated—Binding of anti-CD20 mAb to B cells has been shown to induce tyrosine kinase activation, tyrosine phosphorylation of phospholipase Cγ1, and mobilization of intracellular calcium (16). Further, CD20 mAbs co-precipitate Src family kinases and an unidentified 75–80-kDa phosphoprotein (20), suggesting a mechanism for signal transduction through CD20-associated tyrosine kinase activation. There appear to be at least two distinct epitopes in the small extracellular loop of CD20, one recognized by the antibody 1F5 and another, possibly overlapping epitope, recognized by other CD20 mAbs (24). The 1F5 antibody is unique in its ability to induce c-myc transcription and entry of resting B cells into the G1 phase of the cell cycle, whereas all CD20 mAb, including 1F5, inhibit mitogen-induced activation of B cells (10–15). To explore the molecular basis for the uniquely activating property of the 1F5 anti-CD20 mAb, its ability to co-precipitate kinase activity and tyrosine-phosphorylated proteins was compared with two other CD20 mAbs, 2H7 and B1. Indirect surface immunofluorescence staining demonstrated that all three of these antibodies bind well to Raji cells (Fig. 1). However, there were marked differences in their ability to co-precipitate p75/80 and kinase activity. CD20 complexes precipitated by mAb 2H7 contained more p75/80, as detected by anti-phosphotyrosine, and more kinase activity, as measured by in vitro phosphorylation, than CD20 complexes precipitated by either 1F5 or B1 (Fig. 2). The increased amounts of CD20-associated proteins precipitated by 2H7 might have been partially accounted for by the slightly higher level of binding of 2H7 to the cells (Fig. 1). However, the amount of CD20 precipitated by the 3 mAb was estimated by blotting the same membranes with a CD20 antiserum, and surprisingly, there was significantly less CD20 precipitated by 2H7 than by either 1F5 or B1 (Fig. 2).

**CD20 Becomes Triton-insoluble after Antibody Binding**—The reduced amount of CD20 precipitated by 2H7 mAb was at odds with both the high level of surface staining (Fig. 1) and the higher amounts of CD20 co-precipitated proteins relative to 1F5 and B1 (Fig. 2). These data suggested to us that CD20 may have changed its solubility properties following 2H7 mAb binding, resulting in translocation of CD20 from the Triton-soluble fraction to an insoluble fraction. To test this, Triton-soluble lysates prepared from cells treated with either control or CD20 mAbs were tested for the presence of CD20 by immunoblotting. A significant reduction in the amount of CD20 was detected in lysates prepared from cells pretreated with 2H7 mAbs (Fig. 3, upper panel). The proportion of CD20 that was translocated was estimated to be 80–95% when the cells were exposed to 2H7 for 15 min. In the same time frame, approximately 40–60% and 5–10% of CD20 was lost following exposure to 1F5 and B1 mAbs, respectively. The Triton-insoluble material obtained after centrifugation of the lysates was solubilized in SDS sample buffer and similarly analyzed for the presence of CD20 (Fig. 3, lower panel). When Raji cells were lysed without prior incubation with CD20 mAb, there was no CD20 detectable in the Triton-insoluble fraction. However, addition of either 1F5 or 2H7 CD20 antibody to the cells 15 min prior to lysis resulted in translocation of a significant fraction of CD20 to the insoluble compartment. A much smaller amount of CD20 was translocated in response to B1. The amount of insoluble CD20 varied with the antibody used, in inverse proportion to the amount of soluble CD20 precipitated by the same antibodies. Thus, the 2H7 antibody, which precipitated less soluble CD20 (Fig. 2), was the most effective in inducing CD20 to become insoluble. In contrast, the B1 mAb precipitated the most CD20 from the soluble fraction and was least effective at inducing CD20 insolubility.

The ability of 2H7 to induce CD20 translocation depended on adding the antibody to viable cells prior to lysis; addition of 2H7 to cell-free lysates failed to induce CD20 translocation.
Inverse correlation among CD20 mAbs between precipitation of CD20 and co-precipitation of associated proteins. Raji B cells (10⁷) were incubated with control mAb (CT) or CD20 mAb (1F5, 2H7, B1) for 15 min before lysis. CD20 and associated proteins were precipitated by addition of protein A-Sepharose. Associated proteins were detected by anti-phosphotyrosine blot (upper left) or in vitro kinase assay (upper right). The same membranes were then probed for CD20 (bottom). Note that CD20 migrates as a 33/35-kDa doublet due to differential serine/threonine phosphorylation (41, 42). The lower band is predominant and, at limiting amounts, is the only one detected.

**Fig. 2.** Inverse correlation among CD20 mAbs between precipitation of CD20 and co-precipitation of associated proteins. Raji B cells (10⁷) were incubated with control mAb (CT) or CD20 mAb (1F5, 2H7, B1) for 15 min before lysis. CD20 and associated proteins were precipitated by addition of protein A-Sepharose. Associated proteins were detected by anti-phosphotyrosine blot (upper left) or in vitro kinase assay (upper right). The same membranes were then probed for CD20 (bottom). Note that CD20 migrates as a 33/35-kDa doublet due to differential serine/threonine phosphorylation (41, 42). The lower band is predominant and, at limiting amounts, is the only one detected.

**Fig. 3.** CD20 translocation to Triton-insoluble pellets. Raji B cells were incubated with control mAb (CT) or CD20 mAb (1F5, 2H7, B1) for 15 min before lysis. Both Triton-soluble lysates and Triton-insoluble pellets were analyzed by Western blot for the presence of CD20. Note that there is no CD20 in the insoluble compartment unless cells have been pretreated with CD20 mAb.

(upper panel). 2H7 is capable of binding to CD20 in lysates as shown by its ability to precipitate CD20 from lysates (Fig. 4, lower panel). CD20 translocation into the Triton-insoluble fraction was detected at the earliest time point tested following 2H7 addition, i.e. 15 s, becoming maximal by 15 min and remaining stable for at least 1 h (Fig. 5A). FACScan analysis demonstrated that CD20 molecules which were bound to 2H7 during the initial 15 min incubation remained on the cell surface for at least 1 h (Fig. 5B).

**Fig. 4.** Addition of 2H7 to cell lysates does not induce CD20 redistribution. 5 µg of 2H7 mAb was added to 5 × 10⁶ Raji cells, either before lysis (lanes 1–2) or after lysis (lanes 3–4). Upper panel, Triton-insoluble pellets; Lower panel, CD20 immunoprecipitated from cell lysates. Incubation times for the experiment shown were 30 min (samples in lanes 1–2) and 16 h (samples in lanes 3–4). Similar results were obtained when 2H7 mAb was added to viable cells for 16 h. Samples were separated by SDS-polyacrylamide gel electrophoresis and tested by CD20 immunoblot.

**DISCUSSION**

This report describes the active relocalization of the cell surface protein CD20 into a low density detergent-insoluble membrane compartment upon exposure of Raji B cells to CD20 mAb. Despite the relatively hydrophobic nature of CD20, it is not intrinsically resistant to detergent lysis. All detectable CD20 is found in the Triton-soluble fraction of unstimulated cells and is only induced to become insoluble by the addition to viable cells of mAb against extracellular CD20 epitopes. This phenomenon is not unique to Raji cells since it was observed in several unrelated B cell lines as well as in freshly isolated tonsillar B cells (data not shown). The reason for differences in the degree of insolubility induced by various CD20 mAbs is unclear at present. It is not likely to be due simply to differences in the numbers of CD20 molecules engaged since the B1 mAb binds to CD20 at least as well as 1F5 (Fig. 1), and yet approximately 5-fold more CD20 protein is redistributed by 1F5. It is possible that differences in either affinity or fine specificity account for the observed effects. Antibody binding to CD20 may induce a conformational and/or post-translational modification that causes the molecule to translocate to an insoluble compartment or to become insoluble in situ, perhaps by oligomerization. Alternatively, CD20 may be associated with,

Tyrosine-phosphorylated ~50-kDa Protein Detected Uniquely in the Insoluble Fraction of 1F5-treated Cells—Since CD20 is associated with Src family tyrosine kinases, primarily Lyn, and with a tyrosine-phosphorylated protein, p75/80, the Triton-insoluble membrane fraction in which CD20 was detected was probed by Western blot using anti-lynn and anti-phosphotyrosine. Lyn is one of several Src family kinases enriched in Triton-insoluble membrane compartments (6), and no change in the abundance of Lyn in this compartment was detected upon CD20 translocation (data not shown). Three major bands of approximately 75, 60, and 55 kDa were detected by anti-phosphotyrosine blot in all samples at similar intensity (Fig. 7). The identity of these proteins is not known, but bands in the 55–60-kDa region are likely to include Src family kinases. Of particular interest is that an additional phosphoprotein, migrating at approximately 50 kDa, was uniquely and reproducibly observed in samples pretreated with the activating 1F5 mAb, but not with either 2H7 or B1 (Fig. 7).

REFERENCES

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or be induced to associate with, a GPI-linked protein or other molecule with a predisposition toward localization to the insoluble fraction. GPI-linked proteins are enriched in detergent-insoluble low density compartments (6, 26, 27) thought to represent in vitro isolates of caveolae (3). Under electron microscopy, low density detergent-insoluble fractions can be seen to contain vesicles similar to those formed by caveolae following membrane disruption in Triton X-100 (6, 22, 23) and are enriched in many of the same proteins found in caveolae (see below). Nevertheless, resolution of the issue of whether there are distinct non-caveolar detergent-insoluble microdomains awaits the characterization of additional components in these compartments.

Data is accumulating to implicate these membrane microdomains in the integration of extracellular signals within the plasma membrane (1, 2). In addition to being enriched in cholesterol, sphingomyelin, glycosphingolipids, and GPI-linked proteins, they contain a variety of proteins involved in signal transduction, including Src family tyrosine kinases, G-protein-coupled receptors, calcium channels, and ATPases (28, 29). Caveolae may in fact be major sites for calcium entry/efflux (30, 31), and in this context, it is interesting to note that CD20 appears to be capable of forming calcium channels (17–19).

Further evidence for a role of caveolae or analogous membrane microdomains in signal transduction stems from recent observations that growth factor receptors reside and initiate signaling in these compartments (32–34). However, there are only a few reports describing redistribution of cell surface signaling molecules into these domains. For example, the muscarinic acetylcholine receptor is reversibly translocated into caveolae in response to agonists (35). GPI-linked proteins, which have also been implicated in signaling, become enriched in caveolae upon antibody cross-linking (36, 37). In addition, the high affinity receptor for IgE (Fc\(\varepsilon RI\)) when aggregated, redistributes to detergent-resistant membrane domains (38, 39). CD20 shares significant sequence homology with the \(\beta\) subunit of the Fc\(\varepsilon RI\) complex, particularly in regions corresponding to the putative membrane domains (40). In contrast to activated CD20, which remains insoluble in 1% Triton X-100 (Fig. 5), localization of Fc\(\varepsilon RI\) to membrane domains is extremely sensitive to detergent concentration and is released from the domains in concentrations of Triton X-100 greater than 0.05% (39). Thus, the specific membrane compartment, and/or the mechanisms underlying CD20 and Fc\(\varepsilon RI\) translocation are likely to be different. Furthermore, detergent-insoluble CD20 clearly remains membrane-associated, as assessed by
FACSscan analysis (Fig. 5B), and by confocal microscopy whereas the FcεRI complex becomes internalized (39).

To our knowledge, this is the first description of a lymphocyte cell surface molecule that appears to be completely excluded from detergent-insoluble domains in unstimulated cells but can be induced to translocate to that compartment. The molecular composition of the compartment to which CD20 translocates is presently unknown, but a focus on its components may allow us to identify key downstream elements for CD20-mediated signaling and function. The physiological relevance of CD20 redistribution is suggested by the selective alteration in the profile of tyrosine-phosphorylated substrates in the corresponding compartment induced by the activating mAb 1F5 even though the extent of CD20 redistribution induced by 1F5 is lower than that of 2H7. The activating properties of the 1F5 mAb are thus, for the first time, correlated produced by 1F5 is lower than that of 2H7. The activating properties

3 P. van den Elzen and J. Deans, unpublished observations.