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Lipid Rafts Associate with Intracellular B Cell Receptors and Exhibit a B Cell Stage-Specific Protein Composition

Dirk Mielenz,* Christian Vettermann,* Martin Hampel,† Christiane Lang,* Athanasia Avramidou,* Michael Karas,† and Hans-Martin Jäck*

Lipid rafts serve as platforms for BCR signal transduction. To better define the molecular basis of these membrane microdomains, we used two-dimensional gel electrophoresis and mass spectrometry to characterize lipid raft proteins from mature as well as immature B cell lines. Of 51 specific raft proteins, we identified a total of 18 proteins by peptide mass fingerprinting. Among them, we found vacular ATPase subunits α-1 and β-2, vimentin, γ-actin, mitofilin, and prohibitin. None of these has previously been reported in lipid rafts of B cells. The differential raft association of three proteins, including a novel potential signaling molecule designated swiprosin-1, correlated with the stage-specific sensitivity of B cells to BCR-induced apoptosis. In addition, MHC class II molecules were detected in lipid rafts of mature, but not immature B cells. This intriguing finding points to a role for lipid rafts in regulating Ag presentation during B cell maturation. Finally, a fraction of the BCR in the B cell line CH27 was constitutively present in lipid rafts. Surprisingly, this fraction was neither expressed at the cell surface nor fully O-glycosylated. Thus, we conclude that partitioning the BCR into lipid rafts occurs in the endoplasmic reticulum/cis-Golgi compartment and may represent a control mechanism for surface transport. The Journal of Immunology, 2005, 174: 3508–3517.

Regulated B cell activation by Ag binding to the BCR constitutes the humoral immune response (for review, see Ref. 1). B cell development, characterized by the regulated rearrangement of Ig genes, occurs in the bone marrow and proceeds through several stages, during which the surface expression of BCR alters and reactivity of the BCR is assessed (for review, see Ref. 2). The BCR, consisting of two μ H chains (μHC) and two L chains along with the signal-transducing subunit Igα-Igβ (1), is first expressed at the surface of immature B cells.

To avoid generation of self-reactive B cells, immature B cells harboring a self-reactive BCR can either rearrange their L chain genes for a second time or become anergic or deleted (3). The immature B cells then migrate from the bone marrow to the spleen, where they develop into mature B cells. At this stage, mature B cells are either activated via dual signals from their BCR elicited by Ag binding and a costimulatory T cell or undergo apoptosis when activated by the BCR alone (default response) (4). In experimental systems, anti-BCR μHC F(ab)_2 are frequently used as Ag because they mimic dual signaling of BCR and T cell help in mature B cell lines, such as CH27, as these cells do not undergo apoptosis after this treatment (5).

Conversely, engaging the BCR of immature B cells and immature B cell lines such as WEHI231 by anti-μHC F(ab)_2, induces apoptosis (6, 7). These events have been correlated with the limited entry of the BCR into lipid rafts (5, 8, 9). In contrast, the BCR in mature B cells gains rapid access to lipid rafts after activation with anti-μHC F(ab)_2 (5, 10, 11). Besides activating a B cell or committing it to apoptosis, the BCR functions to deliver foreign Ags to endosomes, where they are processed and loaded as peptide fragments onto MHC class II molecules (for review, see Refs. 12 and 13). Although conflicting data exist about the role of lipid rafts in BCR internalization (14, 15), MHC class II molecules are clearly functionally associated with lipid rafts in APCs (16–18).

The presence of lipid rafts in biological membranes is due to the separation of lipid mixtures into liquid-ordered (Lo/Ld) and liquid-disordered (Ld/Lo) phases according to the content of cholesterol and saturated fatty acids (for review see Refs. 19–21). Lipid rafts, corresponding to Lo domains, can be stabilized by proteins and protein/lipid interactions (19, 22) and be recovered after detergent extraction and sucrose density fractionation in so-called detergent-resistant membranes (DRM) (20).

Since mature and immature B cells differ in their response to BCR ligation (5, 8, 23–25) as well as their ability to stimulate T cells via MHC class II presented Ags (16), we hypothesized that these differences were reflected in the protein composition of DRM. To test this hypothesis, we used two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) to characterize B cell DRM proteins obtained from three B cell lines representing different developmental stages. In this study we demonstrate a differential protein composition of B cell DRM that correlated with the ability of the cells to respond to BCR ligation with apoptosis. Moreover, MHC class II molecules were only detected in DRM of mature, but not immature, B cells. Finally, we provide the first evidence that the cytoplasmic, not fully O-glycosylated, IgM BCR is present in DRM, which leads us to propose that lipid rafts may

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3 Abbreviations used in this paper: μHC, μ H chain; 2D, two-dimensional; DRM, detergent-resistant membranes; ER, endoplasmic reticulum; Lo/Lo, liquid-disordered; Lo/Ld, liquid-ordered; MJ/CD, methyl-β-cyclodextrin; MS, mass spectrometry; PI, phosphatidylinositol; RuBPS, ruthenium bathophenanthroline disulfonate; TR, transferrin receptor; Endo H, endoglycosidase H; IEP, isoelectric point.
regulate molecular traffic in endoplasmic reticulum (ER)/cis-Golgi membranes of B cells.

Materials and Methods

Abs and chemicals
All chemicals were purchased from Sigma-Aldrich or Roth unless stated otherwise. Cell culture medium and medium supplements were obtained from Invitrogen Life Technologies. Affinity-purified goat Abs against mouse μHC were purchased from Southern Biotechnology Associates. Polyclonal rabbit IgG Abs against Lyn (sc-15), vascular ATPase subunit β2 (H180), and G protein β2 (C16) were obtained from Santa Cruz Biotechnology; the mAb against the transferrin receptor (TIR) was purchased from Zymed Laboratories; anti-CD45 mAb was obtained from Cell Signaling; anti-prohibitin mAb was purchased from NeoMarkers; and rat anti-IgG2A coupled to FITC (clone R19-15) was obtained from BD Pharmingen. Rat anti-MHC class II mAb NIMR4 was purchased from Southern Biotechnology Associates, rat anti-MHC class II mAb M5/114.15.2 (26) was obtained from Miltenyi Biotec, and rat mAb R5 (27) against murine VprB was a gift of Dr. M. Cooper (University of Alabama, Birmingham, AL). Polyclonal rabbit Ab against β-actin (20–33) and goat anti-vimentin Ab were purchased from Sigma-Aldrich. Affinity-purified and HRP-conjugated goat Abs against mouse Fcγ were obtained from Jackson ImmunoResearch Laboratories (distributed by Dianova). HRP-conjugated goat Abs against rabbit IgG and goat IgG were obtained from Bio-Rad and Santa Cruz Biotechnology, respectively. To detect Gc1, cholela toxin-HRP (Sigma-Aldrich) was used, and streptavidin-HRP was purchased from Amersham Biosciences. Anti-swiprosin-1 serum was generated by Charles River Laboratories after immunization with a keyhole limpet hemocyanin-coupled swiprosin peptide (C-MEGEGEGATEQPG; gift from Dr. H. Kalbacher, University of Tübingen, Tübingen, Germany) and purified on a GST-Schwann cell receptor column according to standard procedures. The affinity-purified serum was used at a concentration of 0.5 μg/ml for Western blotting.

Cell lines
The murine B cell lines CH27.LX (28), WEHI231 (29), and NYC31.1 (30) were grown in complete RPMI medium (RPMI 1640 containing 2 mM l-glutamine, 1 mM sodium pyruvate, 50 mM 2-ME, 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin) with 5% CO2.

Preparation of DRM
DRM were prepared according to the method described by Cheng et al. (10) with some modifications. Briefly, 106 cells were washed with serum-free, otherwise complete RPMI medium, resuspended at a density of 107 cells/ml, and incubated at 37°C for 30–60 min. Cells were spun down and resuspended in 1 ml of ice-cold TNEV (150 mM NaCl, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1 mM sodium orthovanadate) (10) containing 1% Triton X-100 (w/v), 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin. The detergent/protein ratio was 6/7 (grams/grams) and was roughly the same for each cell line. Cells were lysed on ice for 10 min and further homogenized in a glass/Teflon homogenizer. After centrifugation at 800 × g for 10 min, the supernatant was mixed in 14 × 89-mm polypropylene tubes (Beckman Coulter) with an equal volume of 85% sucrose, overlayed with 6 ml of 35% sucrose, and finally 4 ml of 5% sucrose in TNEV. Sucrose gradients were centrifuged for 16 h at 38,000 rpm in an SW 41Ti swing-out rotor in a Beckman ultracentrifuge with full acceleration and without braking. To harvest gradients, an ice-cooled 60% sucrose solution was applied by a P-500 fast protein liquid chromatography pump (Pharmacia Biotech) into the bottom of tubes fixed in a Brandel fraction collector, and 1-ml fractions were collected from the top. Titration of Triton X-100 from 0.5 to 4% (w/v) did not affect the solubilization of Gc1, Lyn, TIR, or CD45 (not shown). Protein concentrations were determined with a commercial bicinchoninic acid test (Piecer) with BSA as standard.

Gel electrophoresis
One-dimensional SDS-PAGE was conducted according to Laemmli (31) using the Hoefer SE600 system (Amersham Biosciences), and 2D electrophoresis was performed as described previously (32). Briefly, appropriate volumes of DRM or soluble proteins were precipitated with 4 vol of ice-cold acetone for 2–16 h at −20°C. Proteins were collected by a 1-h centrifugation at 10,000 × g and 4°C. Pellets were washed in ice-cold 80% acetone, air-dried, redissolved in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (Merck), 0.5% IPG ampholyte buffer (Amersham Biosciences), 0.001% bromo phenol blue, and 100 mM DTT) and focused after rehydration in 18-cm strips with immobilized pH gradients (IPG pH 3–10) on an IPGphor platform (Amersham Biosciences). Focused samples were subjected to 12% SDS-PAGE using the Hoefer Dalt system according to the manufacturer’s instructions (Amersham Biosciences). The Bio-Rad broad-range marker was used as molecular mass standard.

Staining of 2D gels
2D gels were stained with silver according to Shevchenko et al. (33). Ruthenium II Tris bathophenanthrolin disulfonate (RuBPS) was synthesized as described by Rabilloud et al. (34). Gels were stained with RuBPS as described previously (34) and visualized on a Herolab gel documentation station equipped with UV lamps (λ = 302 nm). 2D gels were scanned and analyzed after calibration with Image Master software (Amersham Biosciences). To detect differences in protein compositions, gels were analyzed manually, and differences were then quantified with AIDA software (Raytest) using several gels (as indicated). Spots were cut out with disposable scalps and stored at −70°C. Isolated, silver-stained protein bands were essentially detergent and essentially digested as described previously (33). RuBPS-stained proteins were digested in 50% ethanol for 2 h (34). Ethanol was removed, and proteins were digested as described previously (33), omitting the first destaining step (34).

Mass spectrometry
Peptide fingerprinting with MALDI-TOF MS was performed on an Applied Biosystems Voyager-DE STR mass spectrometer in the reflector mode after calibration with angiotensin and insulin B chain. Approximately 800-1000 spectra/measurement were added and blasted after internal calibration using trypsin autolysis peaks with Protein Prospector software (http://prospector.ucsf.edu/ucsfhtml4.0/massfti.htm) (35) against current versions of NCBI nr and SwissProt databases. Standard search parameters for Protein Prospector were as follows: minimal numbers of peptides required, 4; p-factor, 0.4; mass tolerance, 50 ppm; missed cleavages, 1; oxidation of methionine factor, 1.0; modification of cysteine, carboxymethylation; modification of methionine, oxidation; contaminating masses, none; and homology mode, identity.

Western blotting
Gel-separated proteins were transferred to nitrocellulose (Schleicher & Schuell). The membrane was blocked in TBST (150 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 0.1% Tween 20) containing 5% nonfat dry milk powder for 1 h. First and secondary Abs (diluted 0.1–1 μg/ml and 1/20,000, respectively) were applied for 1 h at room temperature, followed by four 5-min washes in TBST. Cholera toxin-HRP was diluted 1/20,000. Blots were developed with ECL as described previously (32).

Miscellaneous biochemical methods

Surface biotinylation. Cells (106) were washed repeatedly in PBS and biotinylated with sulfo-LC-NHS-biotin (Pierce) according to the manufacturer’s instructions. Cells were washed three times with PBS containing 10 mM Tris-HCl (pH 7.4) and then lysed. Dead cells were removed by centrifugation through a 2-ml Ficoll cushion before lysis (Lympholyte-M, Cedarlane Laboratories). Immunoprecipitation. Anti-μHC Abs were added to cell lysates prepared with TNEV buffer (10) containing 1% Triton X-100 and protease inhibitors. The mixture was rotated for 1 h at 4°C, 20–50 μl of equilibrated protein G-Sepharose (Pierce) was added, and incubation was performed for 1 h. Immune complexes were collected by short pulse centrifugation, washed three times in lysis buffer, and boiled in 2× SDS sample buffer. Endoglucosidase H (Endo H) digest (36). Immunoprecipitated μHCs were incubated with 100 μl of 0.5% SDS, 100 μg/ml BSA, 50 mM o-DTt, 10 mM Na2HPO4, and 5 mM citric acid (pH 5.0) for 10 min at 95°C. Samples were collected by centrifugation and cooled on ice. One-half of the supernatant was digested with 1 μl of Endo H (Roche) for 2 h at 37°C; the other half was incubated at 37°C without EndoH.

Measurement of lysosomal hexosaminidase activity (37). Cells were centrifuged for 5 min at 200 × g, and the supernatant from 5 × 106 cells was diluted 1/10 in substrate buffer. The reaction was conducted for 1–3 h at 37°C, and OD405 was measured.

Flow cytometry and analysis of apoptosis
Flow cytometry was performed as described previously (36). Briefly, 5 × 106 cells were suspended in FACS-PBS (PBS containing 2% FBS and 0.02% NaN3). Cells were incubated with Abs for 15 min on ice and washed twice with FACS-PBS. For analysis of apoptosis by annexin V staining (38), 105 cells were washed once in annexin V binding buffer (140 mM
NaCl, 2.5 mM CaCl$_2$, and 25 mM HEPES, pH 7.2) and incubated with Cy2-conjugated annexin V (gift from Dr. E. Pöschl, University of Erlangen, Erlangen, Germany) for 20 min at room temperature in a volume of 100 µl. Finally, 400 µl of annexin V binding buffer containing propidium iodide (PI; 0.87 µg/ml) was added, and cells were analyzed immediately. All measurements were performed with a FACSCalibur flow cytometer (BD Biosciences).

Immunofluorescence

Cells were attached to Teflon-coated coverslips (Roth) in serum-free medium for 30 min, stimulated, washed in ice-cold PBS, and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. Cells were then rinsed in PBS and incubated in 3% BSA in PBS. Cells were incubated with Abs, washed in PBS, mounted in Moviol (Hoechst), and analyzed with a Leica confocal microscope after calibration with isotype-matched control Abs.

Results

Isolation of DRM from B cell lines

This study analyzed the protein composition of DRM from B cell lines representing different developmental stages: 1) CH27 mature B cells, a cell line that does not undergo apoptosis after BCR stimulation (5); 2) WEHI231 cells, which represent the immature B cell stage and undergo apoptosis after BCR ligation (7); and 3) NYC31.1 cells, representing an activated B cell line WEHI231 with anti-µHC F(ab)$_2$, only the upper band appeared in DRM fractions of CH27, but not WEHI231 and NYC31.1, BCR ligation (Fig. 1C), in an incubation time that results in maximal stabilization of BCR in DRM (5). We extended our study to the B cell lymphoma line NYC31.1, which is also apoptosis responsive upon BCR ligation (39). Intriguingly, the BCR was also exclusively detected outside DRM of NYC31.1 cells, even upon BCR engagement (Fig. 1D). This indicated a common mechanism underlying distribution of BCR within certain membrane regions and induction of apoptosis.

Notably, after incubation of cells with anti-µHC F(ab)$_2$, only the upper band appeared in DRM fractions of CH27, but not WEHI231 and NYC31.1. This was confirmed by measuring annexin V incorporation into the plasma membrane regions and induction of apoptosis. This study analyzed the protein composition of DRM from B cell lines representing different developmental stages: 1) CH27 mature B cells, a cell line that does not undergo apoptosis after BCR stimulation (5); 2) WEHI231 cells, which represent the immature B cell stage and undergo apoptosis after BCR ligation (7); and 3) NYC31.1 cells, which are also apoptosis responsive upon BCR ligation (39).

Intriguingly, the BCR was also exclusively detected outside DRM of CH27 cells (the same results were obtained for WEHI231 and NYC31.1 cells; data not shown).

2D gel electrophoresis of DRM of B cells

To analyze the protein composition of DRM by 2D gel electrophoresis, we first had to ascertain whether copurified proteins specifically associate with DRM fractions or are contaminants of the detergent-soluble fraction. Because DRM are stabilized by cholesterol, depletion of cholesterol from cells with methyl-β-cyclodextrin (MβCD) (40) is commonly used to control for specific associations of proteins with DRM. Thus, we treated CH27, WEHI231, and NYC31.1 cells with increasing concentrations of MβCD (Fig. 3). All three B cell lines tolerated only low concentrations of MβCD, because 4 mM MβCD resulted in a sharp decrease in Cy2-conjugated annexin V staining in the plasma membrane (Fig. 3A). To prevent obvious destruction of the plasma membrane, DRM association of the marker protein Lyn (10) was unaffected by treatment with 4 mM MβCD (Fig. 3C). Moreover, 2D gels of DRM fractions of untreated vs MβCD-treated cells appeared identical (not shown). In summary, the plasma membrane was at least partially destroyed by MβCD treatment, but DRM were not solubilized. Hence,
FIGURE 2. Immunoprecipitation of surface-biotinylated proteins. A, Lysates from surface-biotinylated CH27 cells were subjected to immunoprecipitation with anti-μHC Abs. Immunoprecipitates (lanes 2 and 3) and an aliquot of the total cell lysate (TCL; lane 1) were subjected to 10% SDS-PAGE, followed by Western blotting. The membrane was probed with HRP-streptavidin to detect biotinylated proteins (lanes 1 and 2) or with HRP-coupled anti-μHC Abs (lane 3) and developed with ECL. Molecular mass standards are shown on the left (kilodaltons). The positions of surface μHC, cytoplasmic μHC, and κ are marked on the right. B, Anti-μHC immunoprecipitates from CH27, NYC31.1, and WEHI231 cells were either treated with Endo H (lanes 2 and 4) or incubated without Endo H at 37°C in digestion buffer (lanes 1 and 3) as indicated at the top. Immunoprecipitates were resolved by 10% SDS-PAGE, followed by Western blotting with anti-μHC Abs. Molecular mass standards are shown on the left (kilodaltons). The positions of the mature and immature μHC are marked on the right.

MβCD treatment appeared inappropriate to assess the specific association of proteins with DRM in the B cell lines used for this study. Therefore, we performed a subtractive 2D gel analysis. Because the DRM fractions were ~99% pure according to distribution of TIR (Fig. 1A), we ran 2D gels in parallel with equal protein amounts from soluble fractions (fractions 10–12) and DRM fractions (fractions 4 and 5). Protein patterns obtained by staining with silver or RuBPS were compared (Fig. 4A). DRM-enriched proteins were detected at high levels as shown in Fig. 4A (e.g., spots 5, 6, 8–14, 18, and 19). The gels in Fig. 4A also demonstrate that protein spots equally abundant in DRM and soluble fractions can easily be identified (see circles in Fig. 4A). With this subtractive method, we identified 51 protein spots in DRM fractions that were not or were hardly detectable in soluble fractions (Fig. 4B).

Identification of DRM proteins by MALDI-TOF MS

To precisely characterize DRM proteins, several 2D gels of DRM fractions were compared with 2D gels of soluble cell fractions. DRM spots 1–51 were excised several times and analyzed by MALDI-TOF MS (Table I). Most of the mass searches had a data error <50 ppm (see Table I). From the 51 spots, 18 proteins could be identified according to the criteria proposed by Thiede et al. (41), who suggested a minimal sequence coverage of 30% for peptides derived from a given protein. This criterion applied for all spots in Table I, except for spots 5, 6, 33, 34, and 41. Nevertheless, determination for spots 5 and 6 was reliable, because rather high MOWSE probability scores were achieved, and a low data error was obtained. Moreover, measured and theoretical molecular mass and isoelectric point (IEP) were almost identical, and the protein spots were located in areas as previously described for 2D gels (42). Spot 5, therefore, probably represents either mitofilin isoform 1 or 2, whereas due to increased IEP and decreased molecular mass, spot 6 is probably isoform 3 (43). Likewise, spots 33 and 41 are depicted in Table I despite sequence coverage <30%. However, they formed part of the pearl-like pattern of spots 32–34 and 39–41 (Fig. 4B), of which two (spots 32 and 40) were identified as MHC class II protein chains, and, moreover, MHC II molecules had matched the submitted peptides (Table I). The discrepancy in expected and observed molecular masses and IEPs for vimentin (spots 47 and 51) is probably due to proteolytic processing of vimentin by caspsases (44). In support of this, one of the vimentin fragments (fragment A; 29.7 kDa; IEP, 5.3) (44) fits almost perfectly to spot 47 (29.9 kDa; IEP, 5.7).

The identified DRM proteins have been arranged in Table I according to the arbitrary numbering given in Fig. 4B. Surprisingly, we realized that many DRM proteins were not derived from the plasma membrane, but, rather, from other subcellular
compartments. This is depicted in Table II, where proteins were arranged according to cellular localization and function. Some proteins, such as prohibitin, MHC class II molecules, and the H9262 HC of the BCR have been assigned to several organelles, whereas others, such as mitofilin, were organelle specific. Importantly, the presence of three mitochondrial proteins in DRM (spots 5, 6, and 48) strongly infers the existence of lipid rafts in mitochondrial membranes. In addition, we identified a novel protein (swiprosin-1, spot 42), which is a potential candidate for DRM-regulated BCR signaling pathways.

Table I. Proteins identified by MALDI-TOF MS in B cell DRM

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<th>Spot No.</th>
<th>MM (kDa)</th>
<th>IEP (pH)</th>
<th>Name</th>
<th>NCBI Accession Number</th>
<th>MOWSE Score</th>
<th>Sequence Coverage (%)</th>
<th>Data Error (ppm)</th>
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<td>105.0</td>
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<td>30</td>
<td>70.0</td>
<td>14</td>
</tr>
<tr>
<td>48</td>
<td>32.3/28.8</td>
<td>5.9/5.6</td>
<td>Prohibitin</td>
<td>6679299</td>
<td>9.5E+04</td>
<td>54</td>
<td>28.0</td>
<td>8</td>
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<tr>
<td>51</td>
<td>29.9/53.7</td>
<td>5.7/5.1</td>
<td>Vimentin</td>
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<td>1.4E+07</td>
<td>35</td>
<td>55.0</td>
<td>17</td>
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</tbody>
</table>

<sup>a</sup> Proteins were isolated from 2D gels, digested with trypsin, and resulting peptides were eluted and analyzed by MS. Emanating from uncleaved proteins, columns 2 and 3 represent measured (meas.) and calculated (calc.) molecular mass (MM) and isoelectric points of the identified proteins, respectively (calculations performed at www.iut-arles.up.univ-mrs.fr/w3bb/dlabim). Measurements represent the mean of three to four analyzed spots.

<sup>b</sup> Included in the table despite sequence coverage below 30% for reasons described in Results.

<sup>c</sup> Name according to SwissProt database.

FIGURE 4. Protein composition of DRM in CH27 B cells. A, Fractions 4 and 5 (DRM) as well as fractions 11 and 12 (soluble) of sucrose gradients from CH27 cells were pooled. Protein (50 µg) were precipitated, subjected to 2D gel electrophoresis, and detected by silver staining. Arrows indicate proteins detected in DRM fractions, and circles mark proteins present in DRM as well as soluble cell fractions. Molecular mass standards are given on the left (kilodaltons). Only parts of the 2D gels are shown. B, Full image of the 2D gel of DRM fractions in A. Numbered arrows indicate proteins detected in DRM, but not soluble cell fractions. Molecular mass standards are given on the left (kilodaltons). Calibration of the first dimension (linear isoelectric focusing; pH 3–10) is marked at the bottom.
Differences in the DRM protein composition between B cell lines

Controversial data exist about the role of DRM in apoptotic BCR signaling in WEHI231 cells (5, 45). However, if DRM play a key role in BCR signaling, then they would also be expected to be involved in apoptotic signals (45). We propose that differences in the protein composition of DRM control the BCR signaling outcome. To test this, we compared DRM of CH27 cells to those of WEHI231 and NYC31.1 cells, both of which undergo apoptosis after engagement of the BCR (7, 39).

Consistent with our hypothesis, the DRM protein pattern differed between CH27 and WEHI231/NYC31.1 cells. For example, spots 8 and 9 (BCR α-HC), 32–34 (MHC class II A α-κ), as well as 39–41 (MHCII E β-κ) from CH27 DRM were not detectable in DRM of WEHI231 and NYC31.1 cells (Fig. 5, A and C, and data not shown). Accordingly, spots 22, 23, and 30 were enriched in CH27 DRM compared with DRM of NYC31.1 and WEHI231 (Fig. 5D and data not shown); however, only spot 23 (γ-actin) could be identified by MS. In contrast, spot 42 (swiprosin-1) appeared to be enriched in DRM of WEHI231 and NYC31.1 cells (Fig. 5B).

To validate our data, we performed Western blotting of DRM fractions as well (Fig. 6). This revealed that 1) each analyzed protein was present in DRM according to Western blot; and 2) the newly identified DRM protein swiprosin-1 is differentially associated with DRM of CH27 and WEHI231 cells. Hence, DRM of various B cell lines differ with respect to BCR α-HC, γ-actin molecules, MHC class II molecules, as well as swiprosin-1. Additionally, two other, as yet unidentified proteins, spots 22 and 30 (Fig. 5), were enriched in CH27 DRM. In addition, our data strongly suggest that the μHC of the BCR (spots 8 and 9; Fig. 5) associates with DRM already in the ER/cis-Golgi of CH27 cells.

In contrast to CH27 cells, MHC class II molecules were not detected in DRM of WEHI231 and NYC31.1 cells. However, flow cytometric analysis using an anti-pan-MHC class II Ab established that WEHI231 cells do express MHC class II molecules at the cell surface at levels comparable to CH27 cells (Fig. 7). To ascertain whether MHC class II molecules are excluded from lipid rafts in the immature B cell line WEHI231, we performed confocal immunofluorescence microscopy (Fig. 7C). Although MHC class II

Table II. Function and subcellular localization of identified proteins*

<table>
<thead>
<tr>
<th>Cellular Compartment</th>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Function in B Cells (selected)</th>
<th>Reference (selected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>32, 33</td>
<td>MHCII A α-κ</td>
<td>Ag presentationb</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>40, 41</td>
<td>MHCII E β-κ</td>
<td>Ag presentationb</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>G protein subunit β2</td>
<td>G protein β2 effectorscd</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>Swiprosin-1</td>
<td>Presently unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Prohibitin (BAP32)</td>
<td>BCR signalingd</td>
<td>60</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>2, 23, 36b</td>
<td>Actin</td>
<td>Endocytosis; signalinga&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>61, 62</td>
</tr>
<tr>
<td></td>
<td>47, 51</td>
<td>Vimentin</td>
<td>Regulation of cell spreading</td>
<td>63</td>
</tr>
<tr>
<td>Endosomes/lysosomes</td>
<td>7</td>
<td>Vacular ATPase subunit α1, isoform 1</td>
<td>Acidification of lysosomes.</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Vacular ATPase subunit β2</td>
<td>Acidification of Lysosomes.</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>32, 33</td>
<td>MHCII A α-κ</td>
<td>Ag presentationc</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>40, 41</td>
<td>MHCII E β-κ</td>
<td>Ag presentationc</td>
<td>12</td>
</tr>
<tr>
<td>ER/Cis-Golgi</td>
<td>8, 9</td>
<td>BCR, μ H chain</td>
<td>Unknown</td>
<td>65, 66</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>5, 6</td>
<td>Mitofilin</td>
<td>Chaperone; regulation of protease activity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Prohibitin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Identified proteins were assigned to cellular compartments and functions according to published data. References apply either to the known function of a given protein in B cells (<sup>a</sup>), to the known function independently of the cellular environment (<sup>b</sup>), or to the putative function in B cells (<sup>c</sup>). Abbreviations: PLCβ, phospholipase C β; Btk, Bruton’s tyrosine kinase.

FIGURE 5. Differential analysis of DRM proteins in B cells. A, Fifty micrograms of DRM proteins of CH27 and WEHI231 cells were applied to 2D gel electrophoresis and then silver-stained. Numbered arrows indicate specific DRM proteins (spots 40–41, MHC II E-β-κ; spot 42, swiprosin-1, spot 37, G protein subunit β2). B, Comparative analysis of the relative abundance of spot 42 (swiprosin) in DRM of CH27, NYC31.1, and WEHI231 cells. 2D gels were scanned, and mean volumes of spot 42 (swiprosin) were divided by volumes of spot 37 (G protein) for each cell line. C, Fifty micrograms of DRM proteins of CH27, NYC31.1, and WEHI231 cells were applied to 2D gel electrophoresis and then silver stained. Numbered arrows indicate specific DRM proteins differing between cell lines (spots 8 and 9, BCR α-HC; spot 23, γ-actin).
and G<sub>m1</sub>, a lipid raft marker detected by cholera toxin subunit B labeling, are clearly colocalized in the mature B cell line CH27, they showed less codistribution in WEHI231 cells.

To further analyze whether MHC class II molecules are physically coupled to G<sub>m1</sub>-containing lipid rafts in CH27 cells, we incubated the cells with anti MHC class II Ab for 20 min before fixation (images marked with /H11001 in Fig. 7C). This showed that G<sub>m1</sub> clearly coclustered and colocalized with surface MHC class II molecules in CH27 cells, whereas MHC class II-bearing membrane regions of WEHI231 cells were clearly separated from G<sub>m1</sub>-containing microdomains.

**Effect of differential DRM partitioning of MHC class II molecules on apoptosis**

Induction of apoptosis through anti-MHC class II Abs is a well-characterized phenomenon and involves presentation of phosphatidylserine on the cell surface (for review, see Ref. 46) that can be detected through binding of annexin V (38). We were interested in whether the induction of anti-MHC class II-mediated apoptosis depends on the presence of MHC class II in lipid rafts. For this purpose, we stimulated CH27 and WEHI231 cells with anti-pan-MHC class II Ab NIMR4 and measured the increase in annexin V-positive cells (Fig. 8A). We did not discriminate between intact (annexin V-positive/PI negative) or disintegrated (annexin V-positive/PI-positive) cells as MHC class II-mediated apoptosis leads to a very rapid disintegration of cells, making it almost impossible
WEHI231 cells were either left unstimulated or stimulated with anti-MHC class II mAbs NIMR4 (5 μg/ml) in 24-well plates for 6 h. Cells were then stained with Cy2-labeled annexin V and PI and analyzed by flow cytometry. A: Quantification of apoptosis induced by anti-MHC class II Ab NIMR4 in CH27 and WEHI231 cells. Data are shown as the fold increase in apoptotic and secondary necrotic cells (annexin V/PI-positive and annexin V-positive cells) and represent six measurements from three independent experiments.

Discussion

In this study we demonstrate for the first time the specific association of vimentin, vacuolar ATPase subunits, mitofilin, and prohibitin with DRM of B cells (Table I). Moreover, we identified a novel protein, swiprosin-1, that appeared to be enriched in the lipid raft fraction of WEHI231 and NYC31.1 cells (Fig. 6A). This is the first time that murine swiprosin-1 has been detected. Only recently, the human homologue of this protein has been described in T cells (48) and HeLa cells (49), but to date it had not been related to DRM. We hypothesize that swiprosin-1 is involved in DRM-regulated transduction of BCR signals. The detailed description and functional analyses of swiprosin-1 will be published elsewhere (our manuscript in preparation) because this would exceed the scope of this report.

Although the function of some of the other proteins described in this study is still speculative with regard to DRM (Table II), the presence of vacuolar ATPase subunits strongly suggests a role for lipid raft-associated enzymes in Ag presentation. Because vacuolar ATPase subunits and Gβ2 have been detected in DRM of both endosomes and Golgi apparatus in CHO and MDCK cells (50, 51), an open question still is: where do DRM proteins actually reside in intact cells? Careful subcellular fractionation before analysis of DRM might be appropriate for future experiments. In line with this, mitofilin, a DRM protein we identified in this study, was probably derived from the inner membrane of mitochondria (Table II). Hence, we hypothesize that DRM may exist in the inner mitochondrial membrane, a possibility we are currently testing in our laboratory using purified mitochondria.

Several groups have analyzed known proteins and signaling pathways in DRM from B cells (5, 10, 11, 45). In contrast to their studies, we did not detect classical signaling molecules in DRM with the proteomic approach, except for Gβ2 (spot 37). Although proximal BCR signaling molecules, such as Lyn, were easily detectable by Western blotting (Fig. 3), the corresponding spots in 2D gels were hardly, if at all, stained with silver (data not shown). Hence, we cannot exclude the possibility that the proteins identified in this study may not represent the complete number of DRM-associated proteins.

During the course of this work, Saeki et al. (52) identified a novel DRM protein, raftlin, in human Raji B cells. Their approach was based on 1D gel electrophoresis, followed by liquid chromatography electrospray ionization MS/MS. Some of the proteins detected by Saeki and colleagues were also described in this study, namely Gβ2, MHC class II molecules, and β-actin (52). In contrast, none of the other proteins that we detected has been reported by Saeki et al. (52), and vice versa.

With regard to cytoskeletal proteins, the description of DRM-associated tubulin by Saeki et al. (52) is contradictory to other reports (10) that described tubulin as a fully soluble protein in B lymphocytes. One explanation might be that in the report of Saeki et al. (52) as well as other reports, no discriminative MS analysis between soluble and DRM factions was performed (52, 53), underlining the benefit of a 2D gel-based subtractive analysis (see Fig. 4).

Another advantage of the established 2D gel map is that, in contrast to one-dimensional gel electrophoresis, quantitative and qualitative differences between B cell lines can be analyzed (see Fig. 5). For example, we showed that MCH class II chains are not present in DRM of WEHI231 cells, although they are expressed at the cell surface (Fig. 7). Since raft association of MHC class II molecules is important for Ag presentation and activation of Th cells (16, 18), our findings point to a new mechanism of B cell tolerance; the inability to organize Ag-presenting MHC class II molecules in lipid rafts could conceivably prevent an immature B cell from being efficiently costimulated by Th cells, which may contribute to clonal deletion or anergy. Particularly, immature transitional B cells can only weakly activate CD4 T cells in contrast to mature B cells (25).

With respect to MHC class II-induced apoptosis, we could not detect a difference between the mature B cell line CH27 and the immature B cell line WEHI231. Thus, in contrast to the BCR (5); raft localization of MHC class II molecules neither positively nor negatively correlates with induction of apoptosis. This finding indicates that the apoptotic pathways used by MHC class II and the BCR may involve different mechanisms. Indeed, in contrast to BCR-induced apoptosis (54), MHC class II-mediated apoptosis does not involve caspases (46).

With regard to regulating the DRM association of the BCR, a direct role for MHC class II molecules appears rather unlikely,
because the BCR and MHC class II molecules form distinct complexes (55). Nevertheless, the transfer of Igα-Igβ from the BCR to MHC class II molecules involves a physical interaction of an unknown nature (55), which could possibly stabilize the BCR in DRM. More likely, however, the increased abundance of γ-actin in DRM of CH27 cells indicated that structural proteins may be involved in stabilizing the BCR/DRM interaction. This is supported by two findings. First, the F-actin-disrupting drug latrunculin A inhibited the DRM association of human BCR transfected in murine A20 lymphoma B cells (56). Second, several spots we identified in this study were cytoskeletal proteins. Among them, spot 2 was double the expected size for γ-actin and may thus represent an urea- and SDS-stable dimer; in addition, spot 36b might be a degradation product of actin.

Finally, somewhat unexpected, the intracellular BCR μHC (spots 8 and 9) was prominent in DRM of CH27, but not in WEHI231 and NY3C31.1 cells (Figs. 1 and 5). However, in sharp contrast, the surface BCR of CH27 cells did not associate with DRM unless cross-linked by anti-μHC F(ab), (Fig. 1) (5, 10). Thus, an intriguing possibility is that the IgM BCR is cross-linked intracellularly, possibly through interactions with chaperones and/or other enzymes that reside in lipid rafts. One candidate molecule is prohibitin (BAP31), which we identified in B cell lipid rafts and which has recently been shown to retain membrane IgD complexes in the ER (57).

It is tempting to speculate that folding, glycosylation, or retention of the BCR occur in intracellular lipid rafts. In this regard, the low levels of the BCR μHC expressed at the surface of CH27 cells (Fig. 1B and data not shown) are consistent with a role for DRM in cytoplasmic retention. Therefore, we hypothesize that partitioning the BCR into DRM can be established in the ER/cis-Golgi compartment of B cells and not exclusively at the cell surface as previously thought. Thus, in analogy to the classical raft hypothesis, lipid rafts in B cells might serve as sorting platforms during protein surface transport in nonpolarized B cells similar to the role they have in polarized epithelial cells (for review, see Ref. 58).

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

References


