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Profiling the Lipid Raft Proteome from Human MEC1 Chronic Lymphocytic Leukemia Cells

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Abstract

Lipid rafts are specialized micro-domains located in the outer plasma membrane of cells and play important roles in various cellular functions, including cell signalling, secretory and endocytic pathways. Cell surface profiling, in particular the lipid raft proteome, has attracted interest in oncology due to the potential use of raft proteins as novel targets for diagnostics and therapeutics. Three different methods have been used to identify the lipid raft proteome from the human chronic lymphocytic leukemia (CLL) cell line MEC1. Firstly, lipid raft proteins were enriched and identified using sucrose gradient ultracentrifugation and 2D liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). To confirm protein localization to the lipid raft, proteomes were compared before and after cholesterol depletion by methyl-β-cyclodextrin (MβCD) using isobaric tags for relative and absolute quantitation (iTRAQ)-labeling coupled to 2D LC-MS/MS. Lipid raft proteins were also identified by immuno-precipitation of cross-linked CD20, a tetraspanin protein that translocates to lipid rafts following treatment with the therapeutic antibody, rituximab. In total, 643 proteins were found in lipid rafts of CLL cells (580 following sucrose gradient ultracentrifugation, 181 depleted by MβCD and 199 isolated by immunoprecipitation) and 64 proteins were identified by all 3 methods). These data represent the first comprehensive profile of the lipid raft proteome in CLL cells and include 30 proteins with no previous known association to the lipid raft. These proteins may represent novel diagnostic and therapeutic targets for CLL.

Keywords: Lipid raft; Chronic lymphocytic leukemia; Methyl-β-cyclodextrin; Immuno-precipitation; Mass spectrometry; Rituximab; Proteomics

Abbreviations: ACN: Acetonitrile; BCR: B-cell Receptor; CAV-1: Caveolin-1; CD: Cluster of Differentiation; CLL: Chronic Lymphocytic Leukemia; ER: Endoplasmic Reticulum; FasR: Fas Death Receptor; GM-1: Monosialo-Tetrahexosyl-Ganglioside; GPCR: G-Protein Coupled Receptor; GPI: Glycosylphosphatidylinositol; HRP: Horseradish Peroxidase; HLA: Human Leukocyte Antigen; Ig: Immunoglobulin; IDA: Information Dependent Acquisition; iTRAQ: Isobaric Tags for Relative and Absolute Quantitation; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; LR: Lipid Raft; MMTS: Methyl Methane Thiosulfonate; MβCD: Methyl--Cyclodextrin; PPI: Protein Phosphatase 1; Rb: Retinoblastoma protein; ROxS: Reactive Oxygen Species; RP: Reverse Phase; SCX: Strong Cation Exchange; SIPA1: Signal-Induced Proliferation-Associated Protein 1; ST6GAL1: ST6 β-galactoside α-2,6-Sialyltransferase 1; TEAB: Triethylammonium Bicarbonate

Introduction

Lipid rafts (LR) were first characterized as functional membrane micro-domains about two decades ago [1]. Although their existence was controversial for many years, investigations of the structure and function of LRs are now at the forefront of cell biology and bio-membrane research [2,3]. LRs are defined as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes [4]. Proteins are recruited to LRs in response to various stimuli, e.g., radiation [5], hormones [6] and therapeutic antibodies [7]. This recruitment is primarily mediated by acylation or farnesylation of a GPI (glycosylphosphatidylinositol) anchor [8]. LRs can act as cellular portals, linking endocytic pathways to the external environment, and enabling internalization of viral particles [9] and toxins [10]. The distribution of LRs or sphingolipid-based micro-domains over the cell surface depends on the cell type. In B-lymphocytes, LRs may be concentrated in microvilli-rich regions [11].

LRs have been implicated in a variety of diseases, including Alzheimer's, prion diseases and cancer [12]. Raft micro-domains provide a platform for various cellular pro- and anti-apoptotic signalling pathways that may be initiated upon LR redistribution [13,14]. Many receptor tyrosine kinases are localized in LRs [15-18] highlighting their importance for cancer signalling. Also many of the surface antigens aberrantly expressed on B-cell chronic lymphocytic leukemia (CLL) cells are localized or translocated to LRs, where they may carry out abnormal functions [19]. This membrane domain is also an important binding site for therapeutic antibodies (e.g., anti-CD20 rituximab and anti-CD52 alemtuzumab) used to treat CLL patients [20,21]. The therapeutic antibody rituximab, binds CD20 on CLL cells and the CD20-rituximab complex is translocated to the LR [7]. This movement increases intracellular Ca^{2+} levels, triggering downstream apoptotic signalling [20]. The therapeutic antibody alemtuzumab, induces caspase-independent apoptosis in CLL cells by cross-linking CD52-enriched LRs [21]. In addition, several anti-cancer drugs suppress growth and induce apoptosis of tumour cells through LR

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remodelling, for example, edelfosine, avicin D, resveratrol and liver X receptors [22]. Due to their functional importance and the potential for identifying new drug targets, protein analyses of this membrane domain are of great interest but the high cholesterol content of LRs makes analysis challenging [23].

Several proteomic studies have profiled the LR proteome and identified proteins with potential clinical importance [24,25]. LC-MS/MS analysis identified 177 proteins in LRs of melanoma cells, with proteins differentially partitioning in LRs of melanoma cells depending on the degree of malignancy [26]. Blonder et al. [27] isolated 380 LR proteins from the Vero cell line, including a large number of hydrophobic integral membrane proteins. Moreover, LC-MS/MS analysis of HL-60 cells identified 147 LR proteins, 74 of which changed in abundance during DMSO-induced differentiation [28]. Arielly et al. [29] identified 407 LR proteins in colorectal cancer clones with a subset of proteins changing in abundance between primary and metastatic disease. A proteome profile of CLL LRs may allow insight into the pathophysiology of this malignancy and identify new functionally relevant proteins that could aid the development of new diagnostics and therapies.

The protein composition of LRs is contentious as contaminating proteins are often co-purified with LR proteins. In a recent solution-based analysis of the LR proteome, the problem of differentiating between LR proteins and contaminants was approached by quantifying LR proteins before and after treatment with the cyclic oligosaccharide, methyl-β-cyclodextrin (MβCD) [30]. LRs are specifically disassembled by the extraction of cholesterol in preference to other lipids using MβCD. Following depletion of LR proteins with MβCD, 165 LR proteins were identified in Hela, 196 in 3T3, and 294 in Jurkat cells [31]. In this study, we aimed to achieve comprehensive coverage of this important membrane micro-domain in CLL cells and identify new LR proteins that may be targeted for novel diagnostic and therapeutic approaches.

The human MEC1 cell line, despite negative CD5 expression, is a good model for B-CLL [32]. MEC1 cells express the same light (κ) and heavy chains (μ, δ) as the parent B-CLL cells. The pattern of Bcl-2 family gene expression in MEC1 cells is shifted toward inhibition of apoptosis, as reported for clinical leukemic B-cells [33]. MEC1 cells have a deletion in chromosome 17p11, one of the most frequent chromosomal aberrations in B-CLL [34]. Several functional abnormalities of B-CLL may be explained by abnormalities of the B-cell receptor (BCR). MEC1 cells and 75% of CLL clinical cases do not express the extracellular Ig-like domain of CD79b, part of the BCR [35]. Although immortalized cell lines are fundamentally different to primary leukemia cells, they have similar features of morphology, immuno-phenotype, karyotype, cytogenetics and molecular characteristics and are therefore suitable *in vitro* models for studying cellular and molecular events in leukemia. MEC1 cells are resistant to rituximab treatment [36].

We profiled the MEC1 LR proteome using 3 different approaches. First, we isolated LRs by exploiting their insolubility in non-ionic detergents [37], followed by sucrose gradient ultra-centrifugation and 2D liquid chromatography and tandem mass spectrometry (2D LC-MS/MS). Second, we quantified LR proteins after the disruption of LR by MβCD treatment using isobaric tags for relative and absolute quantitation (iTRAQ)-labeling coupled with 2D LC-MS/MS. Lastly, LR proteins were identified following treatment of MEC1 cells with the therapeutic anti-CD20 antibody, rituximab. Following rituximab exposure, CD20 translocates to the LR. A lipophilic cross-linker was then used to covalently bind adjacent molecules, thereby creating artificial protein complexes. LR complexes were then enriched and immuno-precipitated with rituximab and again, analyzed by 2D LC-MS/MS. The results from these 3 approaches help define the protein composition of LR in MEC1 CLL cells. LR proteins that induce cell proliferation or resistance to apoptosis may represent new therapeutic targets for treating CLL.

Methods

MEC1 cell culture, drug treatment and apoptosis detection

Human MEC1 CLL cells (American Type Culture Collection 204508/S288c, Manassas, VA, USA) were grown in RPMI 1640 medium (HEPES modification; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum and gentamicin 50 mg/L at 37°C in a non-humidified incubator. For MβCD treatment, MEC1 (3×10^5 cells/ml, 3 biological replicates) were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4*2H_2O$, 2 mM KH_2PO_4, pH 7.4) and re-suspended at a density of 4×10^5 cells/ml in serum-free RPMI. After 1 h, cells were washed and incubated with serum-free RPMI for 1 h at 37°C in the absence (control cells) or presence (treated cells) of 1 mM MβCD (Sigma Aldrich, St. Louis, MO, USA). For rituximab treatment, MEC1 cells were grown in triplicate cultures and treated with rituximab (10 μg/mL; Roche, Basel, Switzerland) for 24 h at 37°C. Following treatment with MβCD or rituximab, cells were harvested by centrifugation (350×g, 5 min, room temperature) for apoptosis assays and isolation of LR as described below. Externalization of phosphatidylserine on cells during apoptosis was quantified by Annexin V-PE binding, while the loss of cell membrane integrity was demonstrated by binding of 7-amino-actinomycin D (7-AAD) to DNA (Annexin V-PE apoptosis detection kit 1, BD Biosciences, San Diego, CA). Cells were analyzed in triplicate with a FAC Scan flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software as previously described [38].

Isolation of LRs

LRs were enriched based on their insolubility in Triton X-100 at 4°C, followed by sucrose gradient ultra-centrifugation. Cells were lysed in cold lysis buffer (25 mM morpholinoethane sulfonic acid pH 6.5, 150 mM NaCl, 1% Na_2VO_4 (w/v), 1% (v/v) protease inhibitor cocktail and 1% (v/v) Triton X-100) and incubated on ice for 30 min. The cells were then homogenized by 10 strokes using a Dounce homogeniser (Kimble/Kontes Glass Co, Vineland, NJ, USA) and mixed with 80% sucrose to produce a 45% sucrose-cell lysate. This mixture was used as the lower portion of a sucrose gradient, then over-laid with 35% sucrose and finally with 5% sucrose, before ultra-centrifugation (186,000×g, 21 h, 4°C). Nine fractions were collected from the top of the tube (Figure 2A) and the proteins were purified by centrifugation using fresh lysis buffer and a 3 kDa cut-off filter (20 min, 13,000×g, 4°C; Millipore, Kilsyth, VIC, Australia). Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer's instructions.

Western blot analysis

Protein fractions 1-9 (Figure 2A) were separated by 12% SDS-PAGE, and transferred to an Immuno-Blot™ PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) at 350 mA for 90 min using a Criterion™ Blotter (Bio-Rad Laboratories). After blocking with 5% skim milk in TPST (25 mM Tris/HCl, 2.7 mM KCl, 137 mM NaCl, and 0.1% (v/v) Tween-20, pH 7.6), the membrane was incubated (4°C, 16

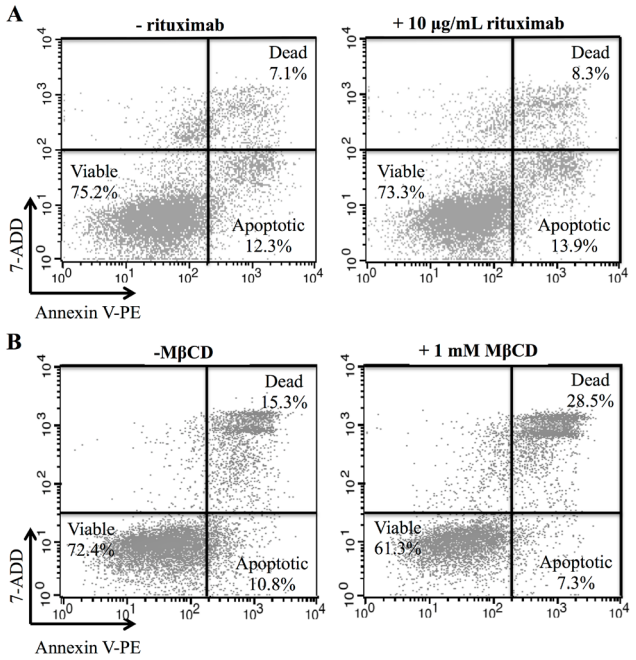

Figure 1: Induction of cell death by MβCD or rituximab. (A) MEC1 cells incubated in the presence (+) or absence (-) of 10 µg/mL of rituximab for 24 h at 37°C and analyzed by flow cytometry. (B) MEC1 cells starved for fetal calf serum for 1 h then incubated in the presence (+) or absence (-) of 1 mM MβCD for 1 h at 37°C. The figures are representative of three independent experiments.

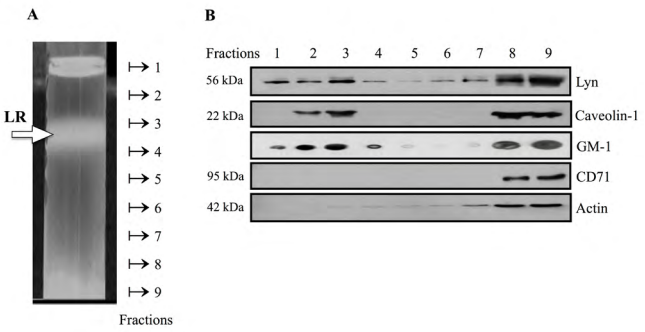

Figure 2: Immuno-blot analysis of lipid rafts isolated from MEC1 cells. (A) Sucrose gradient centrifugation for isolation of LR. The tube was fractionated from top to bottom, an arrow indicates the LR band. (B) Immuno-blot analysis using antibodies against the lipid raft markers Lyn, caveolin-1 and ganglioside M1 (GM1), the plasma membrane marker, transferrin receptor (CD71) and the cytoplasmic marker, actin. The numbers indicate fractions collected. Fractions 1-5 showed the highest levels of lipid raft markers with low levels of markers for the plasma membrane and cytoplasm.

h) with monoclonal antibodies against i) LR markers, anti-caveolin-1 (CAV-1) (BD Biosciences, San Diego, CA, USA) and anti–LYN (Sigma Aldrich, St. Louis, MO, USA); ii) plasma membrane marker, anti-CD71 (transferrin receptor 1; Biolegend, San Diego, CA, USA); and iii) cytoplasmic marker, anti-β-actin (Abcam, Cambridge, MA, USA). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody: goat-anti-mouse-HRP (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or donkey anti-rabbit-HRP (Abcam, Cambridge, CA, USA) for 2 h at room temperature. The LR marker, monosialo-tetrahexosyl-ganglioside (GM-1), was detected by dot blot due to its low molecular weight. Briefly, PVDF membranes were pre-moistened in methanol. Fractions 1-9 (3 µl) were dotted directly onto the PVDF membrane and allowed to dry. The membrane was blocked with 5% skim milk in TPST for 1 h at room temperature and then incubated in peroxidase-conjugated cholera toxin subunit B that binds specifically to GM-1 (Sigma Aldrich, St. Louis, MO, USA) for 1 h. Proteins were visualized using a Rapid Step ECL Reagent (Merck, Whitehouse Station, NJ, USA) and ECL chemiluminescence film (GE Healthcare, Piscataway, NJ, USA).

CD20 immuno-affinity precipitation

MEC1 cells were treated with rituximab (10 µg/mL) at 37°C for 24 h Proteins were then cross-linked by the addition of 2 mM disuccinimidyl tartrate (DST; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at room temperature and the reaction was quenched with 1 M Tris-HCl pH 7.4. DST is a lipophilic, membrane-permeable cross-linker that allows intra-membrane protein conjugation. The spacer arm of this molecule is 6.4 Å and covalently binds primary amines on adjacent molecules, thereby creating artificial protein complexes in LRs. Cells were lysed using Triton X-100 and protein concentrations were determined as previously described. The protein concentration was adjusted to 400 µg/mL with lysis buffer and incubated with Dynabeads® Protein G (Invitrogen, Carlsbad, CA, USA) at 4°C with rotation for 12 h. LR proteins in complex with CD20 were then precipitated following the manufacturer's protocol.

Protein identification by 2D LC-MS/MS

Lysis buffer and sucrose solution containing purified LR proteins was exchanged to 0.5 M triethylammonium bicarbonate (TEAB) pH 8.5, 0.1% SDS (w/v) using a 3 kDa cut-off filter. Proteins were then reduced with 10 mM tris-(2-carboxyethyl) phosphine for 60 min at 60°C, alkylated with 10 mM methyl methane thiosulfonate (MMTS) for 10 min at RT, before digestion with sequencing-grade trypsin (1:25, trypsin to protein; Promega, Madison, MA, USA) at 37°C for 16 h. Digested peptides were dried in a Vacuum Centrifugal Concentrator 5301 (Eppendorf, Hamburg, Germany), re-suspended in 5 µL 1 M TEAB, pH 8.5, and labelled with iTRAQ 4-plex reagents (AB SCIEX, Foster City, CA, USA) following the manufacturer's protocol. Samples were labelled as follows: 114, untreated sample 1; 115, untreated sample 2; 116, 1 mM MβCD-treated sample 1; 117, 1 mM MβCD-treated sample 2. Labelled samples were combined and cleaned using an ICAT cartridge (AB SCIEX) and desalted using a HLB column (Waters, Milford, MA, USA) following the manufacturer's instructions. Labelled peptide mixtures were then separated by strong cation exchange (SCX) chromatography (ZORBAX Bio-SCX series II, 3.5 µm, 50 x 0.8 mm, 300 Å pore size, Agilent, Palo Alto, CA, USA) on an Agilent 1100 HPLC system (Agilent) interfaced with a QSTAR Elite mass spectrometer (AB SCIEX) in on-line mode. Labelled peptides (20 µg) were loaded onto the column at 10 µL/min in SCX buffer A (5% (v/v) ACN, 0.05% (v/v) formic acid, pH 2.5) and eluted into 7 fractions with intermittently increasing salt concentrations to 0%, 2.5%, 5%, 7.5%, 10%, 15%, 20% and 100% SCX buffer B (5% (v/v) ACN, 0.5 M ammonium formate, 2% (v/v) formic acid, pH 2.5). Each fraction was loaded onto a C18 trap column (ZORBAX 300SB-C18 column, 0.3×5 mm, 5 µm particle size, 300 Å pore size, Agilent) at 10 µL/min and washed for 7 min with SCX buffer A before switching the trap column in line with the C18 separation nano-column (ZORBAX 300SB-C18 column, 0.1×150 mm, 3.5 µm particle size, 300 Å pore size, Agilent).

The peptides were eluted directly into the ionization source of the mass spectrometer at 0.6 µL/min with the following gradient: 0 min, 5% reverse phase (RP) solvent B (0.1% (v/v) formic acid in ACN); 8 min, 5% B; 10 min, 15% B; 90 min, 30% B; 105 min, 60% B; 115 min, 5% B; 120 min, 5% B; where RP solvent A was 0.1% (v/v) formic acid in Milli-Q-water. Data acquisition was performed in an information dependent acquisition (IDA) mode using Analyst QS 2.0 software (AB SCIEX). In IDA mode, a TOF-MS survey scan was acquired (*m/z* 350-1750, 0.5 s), with the 3 most abundant multiply charged ions (2+ to 4+, threshold counts >30) in the survey scan were sequentially subjected to product ion analysis. Product ion spectra were accumulated for 2 s in the mass range *m/z* 100-1800 with a modified Enhance All mode Q2 transition setting favoring low mass ions so that the iTRAQ reporter ions (114-117) intensities were enhanced for quantification. Automatic collision energy and automatic MS/MS accumulation modes were used in the advanced IDA settings. Data acquisition was performed with an exclusion of 30 s for previous target ions (50 ppm mass tolerance).

Data analysis

iTRAQ 2D LC-MS/MS data were analysed using Protein Pilot 3.0 software (AB SCIEX). All MS/MS spectra were searched against a combined Swiss-Prot protein database, version uni-sprot 2011. Parameters set in Protein Pilot 3.0 included (1) sample type, iTRAQ 4-Plex, (2) cysteine alkylation, MMTS, (3) digestion, trypsin, allowing 2 missed cleavages, (4) species, *Homo sapiens*, (5) instrument, QSTAR Elite. The following processing options were used: quantitative, bias correction, background correction, biological modifications and thorough identification search. A concatenated target-decoy database search strategy was employed to estimate the rate of false positives. Only proteins identified with at least 95% confidence and unused Prot scores >1.3 were reported. Ratios of peak areas of the iTRAQ reporter ions were used to determine the relative abundances of the peptides.

The results obtained using the Protein Pilot 3.0 software were exported to Microsoft Excel for further analysis. A two-tailed student t-test was performed assuming equal variances between the control and MβCD-treated samples. The average ratios of control to MβCD samples were calculated and used to determine the identified protein levels following MβCD treatment. A decrease in protein level by a ratio of 0.75 or more with $p<0.05$ was considered significant (Table 1 and Supplementary Table 1) and indicated successful depletion by MβCD and therefore localization to the LR.

Results

Effects of MβCD and rituximab on cell viability

MβCD decreased cell viability in a concentration-dependent manner. A low MβCD concentration (1 mM) was chosen for subsequent studies. At this concentration, MβCD efficiently removed cholesterol, with minor effects on cell viability (~10% decrease). No significant increase in apoptosis (control 12.3%, rituximab 13.9%) or necrosis (control 7.1%, rituximab 8.3%) was observed when cells were incubated with rituximab (10 µg/mL, 24 h; Figure 1), as expected. Similarly, MβCD treatment (1 mM, 1 h) did not induce apoptosis (control 10.8%, MβCD 7.3%). However, MβCD induced some necrosis (control 15.3%, MβCD 28.5%; Figure 1).

LR isolation

The LR and associated proteins were isolated from the 5-30% sucrose interface, whereas remaining cellular material, including components of solubilized membranes and other cellular constituents remained in the higher density sucrose layers (i.e., 45%; Figure 2A). Western and dot blot analyses assessed the purity of the LR fractions using 3 established LR markers, CAV-1, LYN and GM1. CD71 and β-actin were used as non-LR associated cell surface and cytoplasmic markers, respectively. As shown in Figure 2B, LR were enriched in the low-density fractions 1–5 of the sucrose gradient indicated by a high abundance of CAV-1, LYN and GM1. LR marker proteins were also abundant in the high-density fractions 8-9, suggesting an incomplete denaturation of LR-associated proteins by Triton X-100. CD71 and β-actin were not detected in the LR fractions (fractions 1-5) indicating successful enrichment of the LR.

Identification of LR proteins enriched by sucrose gradient ultra-centrifugation

2D LC-MS/MS analyses of LR proteins isolated from fractions 1-5 of the sucrose gradient resulted in the identification of 580 proteins (Supplementary Material, Table IV). We used BIOMYN (www.biomyn.de), Uni Prot (www.uniprot.org) and the Human Protein Reference Database, HPRD, (www.hprd.org) to categorise the identified proteins by sub-cellular localisation and biological function (Figure 3A). Proteins were predominantly localized to the plasma membrane (39%), cytoplasm (29%) and mitochondria (13%), and involved in

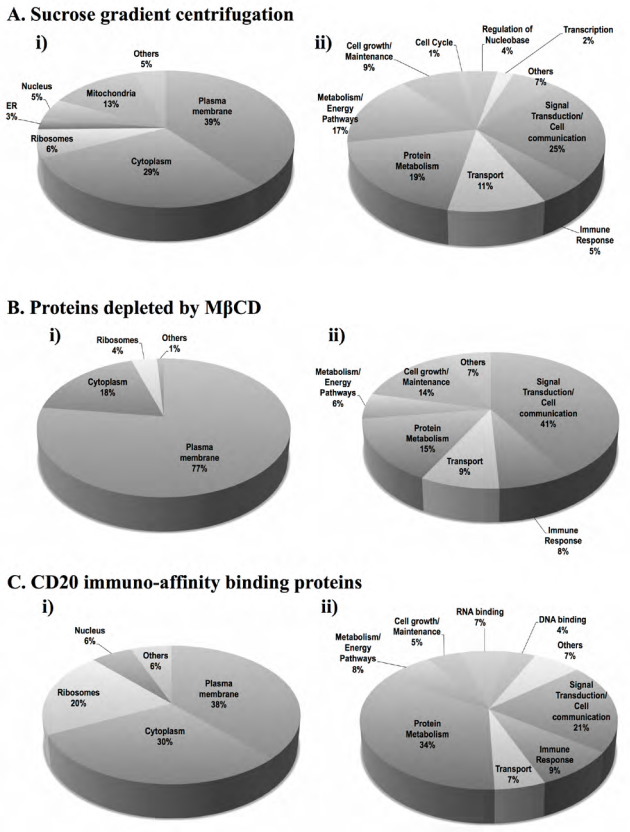

Figure 3: Sub-cellular localization and biological processes of lipid raft proteins from MEC1 cells identified by LC-MS/MS. i) Sub-cellular localization, and ii) biological processes based on the Human Protein Reference Database (www.hprd.org), PubMed (www.uniprot.org) and BioMyn (www.biomyn.de). (A), proteins isolated using sucrose gradient centrifugation (B), proteins identified by MβCD-treatment (C), and proteins identified from LR following immuno-precipitation of CD20 with rituximab.

signal transduction/cell communication (25%), protein metabolism (19%), energy metabolic pathways (17%), transport (11%), cell growth/maintenance (9%) and immune response (5%). Although most are known LR or LR-associated proteins, 20 identified proteins have no known association with the LR and are detailed in Table 1. Most of these novel LR proteins are involved in signal transduction (57%) and immune response (24%). The isolation of LR proteins was highly reproducible using this method; 3 independent sample preparations achieved similar numbers of identified proteins (505-513) with 92-93% overlap (Supplementary Material, Table II).

LR proteins identified following cholesterol depletion

Comparative proteomic analysis of MEC1 cells before and after MβCD treatment identified 181 proteins with decreased abundance (Supplementary Material, Table I; $p<0.05$). Of these, 153 (85%) were also identified by the sucrose gradient enrichment method (Figure 4) and 30 are novel to the LR (Table 1). Proteins depleted by MβCD were categorized predominantly as signal transducer proteins (41%) that are largely confined to the plasma membrane (77%; Figure 3B). Using web-based tools (BIOMYN, Uniprot and HPRD), we examined the post-translational modifications that may target each protein to the LR. The majority of proteins (71%) depleted by MβCD contain myristoylation, palmitoylation, prenylation, acetylation and glycosylation modifications (Table 1 and Figure 5). The depletion of LR proteins by MβCD was highly reproducible between the 3 biological replicates (Supplementary Material, Figure 1).

Immuno-precipitation of CD20-binding LR proteins

2D LC-MS/MS analysis of immuno-precipitated CD20-binding proteins following their translocation with CD20 to the LR induced by rituximab treatment, resulted in the identification of 199 proteins (Supplementary Material, Table V). Of these, 164 (82%) were also identified in the sucrose gradient fractions 1-5, and 64 (32%) were differentially abundant following MβCD treatment (Figure 4). Six proteins identified by IP are novel to LR (Table 1). Overall, 64 (32%) CD20-binding proteins were common to all 3 methods. Thirty-five proteins (18%) were identified by the CD20 IP method alone. Classification using BIOMYN, Uniprot and HPRD, confined proteins mainly to the plasma membrane (38%), cytoplasm (30%) and ribosomes (20%) and with biological functions predominantly relating to protein metabolism (34%) and signal transduction (21%; Figure 3C). The CD20 IP method was highly reproducible; 3 independent sample preparations yielded similar numbers of proteins (185-190) with 95-96% matching across the replicates (Supplementary Material, Table III).

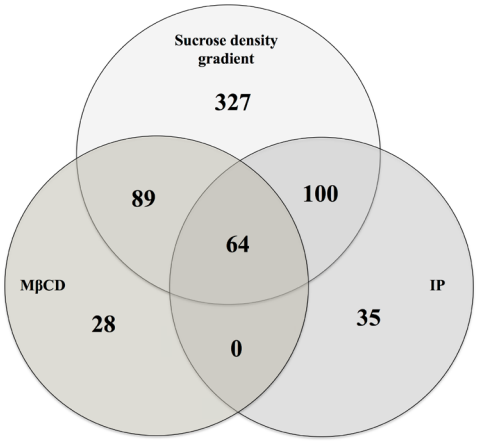

Figure 4: Venn diagram showing the numbers of proteins identified from the three isolation methods for lipid raft proteins. Proteins (580) identified from sucrose gradient fractions (total lipid raft); 181 proteins depleted by MβCD treatment, and 199 isolated by CD20 immuno-precipitation with rituximab; 64 proteins were common to all 3 methods.

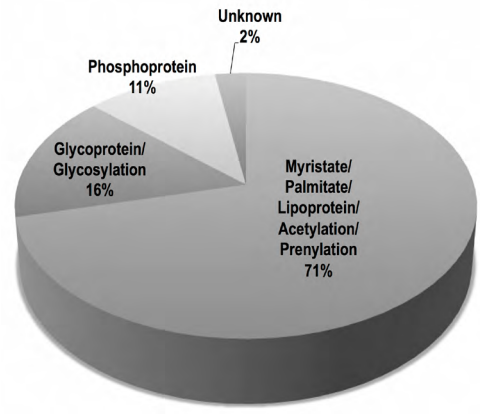

Figure 5: Post-translational modifications of proteins identified in lipid rafts from MEC1 cells following MβCD treatment. The modifications were annotated from the Human Protein Reference Database (www.hprd.org), PubMed (www.uniprot.org) and BioMyn (www.biomyn.de). The percentages of modifications are indicated.

Overall, 643 proteins were identified from the LR of human MEC1 CLL cells, including 30 proteins with no previous LR associations (Table 1). Of these, 6 novel LR proteins, lymphocyte specific protein (LSP1), HLA class II histocompatibility antigens (HLA-DQB1, HLA-DQA1/DRA, HLA-DRA), Ras-related C3 botulinum toxin substrate 2 (RAC2) and Ig kappa chain V-IV region B17 were identified by all 3 methods. These proteins have roles in cell migration, adhesion, signalling pathways, apoptosis, transcription regulation, protein synthesis and degradation.

Discussion

Interactions between the LR and the cytoskeleton are essential for many cellular processes such as signal transduction, endocytosis, cell adhesion and motility and immune responses. LR proteins identified here are involved in processes important in cancer including cell survival, apoptosis and immune recognition and response and are discussed below.

LR proteins involved in apoptosis

LSP1, a novel LR protein isolated by all 3 enrichment methods, is associated with the cytoplasmic face of the plasma membrane and the cytoskeleton (F-actin), and is a marker for human leukocytes. LSP1 is pro-apoptotic in normal immature B-cells by regulating a Ca^{2+}-dependent step in the induction phase of anti-IgM induced apoptosis [39]. Expression of the C-terminal residues 179-330 of the LSP1 protein inhibits anti-IgM-induced translocation of PKCβI to the plasma membrane and activation of ERK2, leading to increased apoptosis [40]. Therefore, blocking LSP1 in the LR before IgM stimulation could result in cell apoptosis. Other apoptosis-related LR proteins identified include SH3KBP1-binding protein 1, a calcineurin B-like-interacting protein

Acc. # [i.]	Protein Name	Gene	Peptide (% Cov) [ii.]	Ratio (p-value)[iii.]	IP[iv.]	SDG[v.]	Primary localisation[vi.]	Modifications[vi.]
Proteins involved in signal transduction								
P33241	Lymphocyte-specific protein 1	LSP1	15(50.4)	0.20 (0.01)	+	+	Plasma membrane	Acetylation
B1AH80	Ras-related C3 botulinum toxin substrate 2	RAC2	3 (17.3)	0.40 (0.04)	+	+	Plasma membrane	Acetylation/ prenylation
P01919	HLA class II histocompatibility antigen, DQ(W1.1) beta chain	HLA-DQB1	8 (36.8)	0.54 (0.04)	+	+	Plasma membrane	Glycosylation
P01903	HLA class II histocompatibility antigen, DR alpha chain	HLA-DRA	6 (11.4)	0.30 (0.00)	+	+	Plasma membrane	Glycosylation
P05536	HLA class II histocompatibility antigen, DQ(W3) alpha chain	HLA-DQA1	2 (7.9)	0.54 (0.01)	-	+	Plasma membrane	Glycosylation
Q96SB3	Neurabin II	PPP1R9B	4 (10.2)	0.52 (0.01)	-	+	Plasma membrane	Acetylation
Q9Y4H4	G-protein-signaling modulator 3	GPSM3	2 (23.1)	0.50 (0.03)	-	+	Plasma membrane	Phosphorylation
Q8WU40	Calcium/calmodulin-dependent protein kinase type II subunit gamma	CAMK2G	1 (5.3)	0.30 (0.03)	-	+	Plasma membrane	Phosphorylation
Q92928	Putative Ras-related protein Rab-1C	RAB1C	2 (12.4)	0.62 (0.02)	-	+	Plasma membrane	Prenylation/ acetylation
Q8TBC3	SH3KBP1-binding protein 1	SHKBP1	1 (7.6)	0.40 (0.05)	-	+	Plasma membrane	Phosphorylation
Q01518	Adenylyl cyclase-associated protein 1	CAP1	3 (12.0)	0.51 (0.01)	-	+	Plasma membrane	Acetylation
P01871	Ig mu chain C region	IGHM	8 (19.1)	0.48 (0.05)	-	+	Plasma membrane	Glycosylation
P15907	Beta-galactoside alpha-2,6-sialyltransferase 1, B-cell antigen CD75	ST6GAL1	1 (5.4)	0.46 (0.04)	-	+	Plasma membrane	Glycosylation
Q96FS4	Signal-induced proliferation-associated protein 1	SIPA1	1 (3.5)	0.24 (0.01)	-	-	Cytoplasm	Phosphorylation
Q7LGA3	Heparan sulfate 2-O-sulfotransferase 1	HS2ST1	1 (10.4)	0.54 (0.04)	-	-	Plasma membrane	Glycosylation
P61952	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11	GNG11	1 (28.8)	0.28 (0.00)	-	-	Plasma membrane	Prenylation/ lipid anchor
P09486	Basement-membrane protein 40	SPARC	1 (8.5)	0.50 (0.05)	-	-	Plasma membrane	Glycosylation
Proteins involved in immune response								
A9YLN4	Tyrosine-protein kinase receptor	CD74/ROS fusion	2 (4.8)	0.65 (0.01)	-	-	Plasma membrane	Phosphorylation
P06314	Ig kappa chain V-IV region B17		2 (13.4)	0.69 (0.05)	+	+	Plasma membrane	-
Q7YPT8	MHC class II antigen	HLA-DQA1/DRA	6 (11.4)	0.39 (0.00)	+	+	Plasma membrane	Glycosylation
Q0KKI6	Immunoglobulin kappa light chain	IGKC	4 (28.9)	0.61 (0.03)	-	+	Plasma membrane	-
P13284	Gamma-interferon-inducible lysosomal thiol reductase	IFI30	1 (5.2)	0.24 (0.00)	-	-	Plasma membrane	Glycosylation
P04440	HLA class II histocompatibility antigen, DP(W4) beta chain	HLA-DPB1	3 (26.4)	0.29 (0.00)	-	-	Plasma membrane	Glycosylation
Proteins involved in protein metabolism								
Q6NYC8	Phostensin	PPP1R18	2 (6.7)	0.43 (0.02)	-	+	Plasma membrane	Phosphorylation
Q8WWI1	LIM domain only protein 7	LMO7	3 (4.1)	0.28 (0.01)	-	+	Cytoplasm	Phosphorylation
P29144	Tripeptidyl-peptidase 2	TPP2	2 (2)	0.35 (0.04)	-	-	Plasma membrane	Acetylation
Transport proteins								
Q10567	AP-1 complex subunit beta-1	AP1B1	1 (4.3)	0.46 (0.00)	-	-	Plasma membrane	Acetylation
Q9H0X9	Oxysterol-binding protein-related protein 5	OSBPL5	1 (1.4)	0.30 (0.04)	-	+	Cytoplasm	Phosphorylation
ATP-binding protein								
Q6S8J3	POTE ankyrin domain family member E	POTEE	9 (8.6)	0.46 (0.01)	-	+	Plasma membrane	Phosphorylation
Protein involved in cisplatin resistance								
Q6UW68	Transmembrane protein 205	TMEM205	1 (13.8)	0.42 (0.03)	-	-	Plasma membrane	Acetylation

i. Accession numbers retrieved from UniProt database.
ii. Number of peptides identified with >95% confidence, protein sequence coverage percentage in brackets.
iii. Protein level ratio (all proteins depleted after MβCD treatment) with p-value in brackets.
iv. '+' denotes identification of protein in immuno-precipitation MS/MS analysis.
v. '+' denotes identification of protein in sucrose density gradient MS/MS analysis.
vi. Primary sub-cellular localisation and post-translational modifications were obtained from the Human Protein Reference Database (http://www.hprd.org/

Table 1: Novel lipid raft proteins identified in MEC1 cells by cholesterol depletion using MβCD (1 mM, 1 h).

that enhances tumor necrosis factor mediated apoptosis [41] and regulates epidermal growth factor receptor activation, an important anti-cancer target [42]. Signal-induced proliferation-associated protein 1 (SIPA1), a specific GTPase activator protein was also identified in the LR proteome of CLL cells. Overexpression of SIPA1 in hematopoietic cells including chronic myeloid leukemia (CML) cells inactivates Ras-related regulatory protein RAP1, resulting in reduced pro-survival Erk signalling [43].

G-protein-signalling modulator 3 (GPSM3) localizes to the plasma membrane of the human acute monocytic leukemia cell line, THP-1, and regulates the assembly and function of G-protein heterotrimers (Gα-GDP/Gβγ), that control G-protein coupled receptors (GPCR) at the cell surface [44-46]. Thus, GPSM3 can modulate GPCR signaling, leading to the progression of many cancers and their spread to distant organs [47]. We have identified several members of the heterotrimeric G proteins complex previously documented in LR proteomes, i.e. GNAI2, GNAI3, GNB2, GNB1, and GNB2L1 [31,48]. We also identified GNG11 and modulator GPSM3, that are novel to the LR. Interestingly, the over-expression of GNG11 immediately induces cellular senescence in normal human fibroblasts [49]. Targeting LR proteins involved in apoptosis and/or their modulators may enhance apoptosis of CLL cells.

LR proteins involved in immune response and antigen presentation

RAC2 is a plasma membrane-associated small GTPase [50] that is novel to the LR proteome, identified by all 3 methods here. Activated RAC2 interacts with Rip kinases to drive the immune response, triggers NF-κB and interferon regulatory pathways [51], and is required for IFN-γ production during normal T-cell activation and differentiation [52]. Overexpression of RAC2 has been reported in head and neck squamous cell carcinomas and brain tumors [53]. Many studies implicate RAC GTPases in various aspects of tumorigenesis and as an anti-cancer target for drug development [54]. In human neutrophils, RAC2 translocation from the cytosol to the plasma membrane is necessary to prevent reactive oxygen species (ROxS) production and activation of the mitogen-activated protein kinase pathway [55]. Therefore, disruption of LR or prevention of RAC2 translocation to LR may enhance ROxS production and trigger apoptosis.

CD75 or ST6 β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) has increased expression on B-CLL cells [56] and regulates the expression of differentiation antigens HB-6, CDW75, and CD76 on lymphocytes [57]. The mechanism by which ST6GAL1 aids tumor progression is not clear, but sialylation of the Fas death receptor (FasR) by ST6GAL1 inhibits Fas-mediated apoptosis in colon carcinoma cells [58]. The FasR is localized in LR [59] and is essential for the execution of Fas-mediated apoptosis [60]. Therefore, co-localization of ST6GAL1 to the LR of CLL cells may function to inhibit Fas-mediated apoptosis, therapeutic blockage of ST6GAL1 may enhance Fas-mediated apoptosis in CLL.

CD74-ROS, a fusion of the N-terminus of CD74 with receptor tyrosine kinase ROS, was identified in LR of MEC1 cells. ROS is an important modulator of signal transduction pathways that regulate cellular proliferation, differentiation, migration and survival [61] and the CD74/ROS fusion protein was shown to drive proliferation and survival of a sub-group of non-small cell lung cancers [61,62]. CD74 associates with the major histocompatibility complex, human leukocyte antigen (HLA) class II, and facilitates peptide presentation [63]. HLA class II antigens bind peptides derived from antigens for recognition by CD4 T-cells [64], a crucial part of the anti-tumoral immune response. The HLA class II gene includes highly polymorphic HLA-DR, DQ and DP genes [65]. HLA-DQB1, HLA-DQA1/DRA and HLA-DRA were identified in MEC1 LRs by all 3 methods and are novel to the LR proteome. HLA-DQB1 and HLA-DQA1 form the DQ heterodimer, a cell surface receptor for antigen presentation. In B-CLL up-regulation of HLA-DQA1 is correlated to apoptosis resistance in response to DNA damage [66]. In addition, the HLA-DQA1 gene harbors a susceptibility locus for risk of familial CLL [67]. HLA-DQB1 is also associated with a higher risk for CLL [68] and is a susceptibility allele in acute lymphoblastic leukemia [69]. Localization of HLAs to LRs may initiate tyrosine kinase signaling pathways in B-cells [70] and enable access to the endocytic/exogenous route.

Ig kappa chain V-IV region B17, a protein encoding a variable region associated with the antigen-binding site, was identified in LR of CLL cells using all 3 methods. The immunoglobulin (Ig) heavy and light chains are produced by B-cells to neutralize foreign antigens, and are secreted from cells or bound to the BCR complex [71]. In CLL, secreted Ig and abnormal serum free light chain (kappa and lambda) ratios are associated with poor survival [72,73]. Membrane-bound IgM induces phosphorylation of CD79a and CD79b by Src family kinases, including Lyn [74], that are concentrated in LRs and may facilitate phosphorylation of the LR-localized BCRs [75]. Blocking these Igs could inhibit CLL progression. In addition, Ig kappa chain V-IV region B17 could interfere with the function of therapeutic antibodies, e.g., rituximab, that once bound, translocates to the lipid raft with CD20. The presence of several proteins involved in antigen processing in LRs may provide possible avenues for enhancing an immune response against CLL.

LR proteins involved in cell survival

Neurabin II, a protein phosphatase 1 (PP1) regulatory subunit, is a scaffold protein localized to the cell membrane that interacts with actin [76,77]. In this study, neurabin II (PPP1R9B), PP1 catalytic subunit A (PPP1CA), protein phosphatase 1G (PPM1G) and PP1 regulatory subunit 18 (PPP1R18) were identified in the LR of MEC1 cells by sucrose gradient fractionation and MβCD treatment (Table 1 and Supplementary Table I). PP1 regulates cell cycle progression by activating the growth suppressor retinoblastoma protein (Rb) [78] and conversely, promoting cell survival by dephosphorylating p53 and negatively regulating the p53-dependent death pathway [79]. Neurabin II suppresses growth by inhibiting PP1 activity [80]. Loss of Neurabin II correlates with reduced PPP1CA that maintains elevated pRb and contributes to an increase in p53 activity to arrest cellular proliferation [81]. However, in the absence of p53, reduced neurabin II promotes tumorigenesis [82]. MEC1 cells possess a truncated form of p53 that may be non-functional [83]. Increased abundance of the protein phosphatases and regulators in LR, like neurabin II, PPP1CA, PPM1G and PPP1R18, may trigger a tumor suppressor signal via Rb providing a novel therapeutic approach for p53-mutated CLL.

Proteins identified here possess post-translational modifications such as GPI-anchors, fatty acylation and lipids, necessary to target proteins to LR [37,82,84-87]. Identified GPI-anchor proteins such as BST2, SEMA7A, CD58 and PHB, are built on phosphatidylinositol that inserts into the exoplasmic leaflet of the membrane. Some of these proteins including LSP1, FLOT2, GNB2 and FMNL1 contain alkyl-acyl groups and fatty acylation (myristoylation, palmitoylation, prenylation and acetylation). Ribosomal subunits, RPS16, RPS2 and RPS11 may be targeted to LR-enriched membranes through protein acylation [53]. Lipoproteins such as GNAI2, LYN, FGR, RPS11, EFNA5 and RFTN1 are post-translationally modified by attachment of at least one lipid or fatty acid, e.g., farnesyl, palmitate or myristate [88] that may direct these proteins to the LR [89,90]. *N*-glycosylated proteins, such as SPTBN1, HLA-DPB1, SEMA7A, ST6GAL1 and HYOU1 are important integral membrane and LR proteins [91-94]. The post-translational modifications listed in Table 1 may attach these proteins to LR. A number of phosphorylated proteins were identified, including CSNK1G3, SHKBP1, CAMK2A and LIMA1, and may be important regulators of downstream signaling cascades.

Conclusions

We have identified 643 unique proteins from MEC1 LR using 3 different enrichment methods. Thirty proteins identified had no previous association to the LR (Table 1). Six of these LR proteins (LSP1, HLA-DQB1, HLA-DQA1/DRA, HLA-DRA, RAC2 and Ig kappa chain V-IV region B17) were isolated and identified following enrichment by all 3 methods. These proteins have roles in cell migration, adhesion, signalling pathways, apoptosis, transcriptional regulation, protein synthesis and degradation in cancer cells. Following further analysis in CLL, newly described proteins in this important plasma membrane sub-region, may represent new therapeutic targets for the treatment of CLL.

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