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Protein expression profiling of CLL B cells using replicate off-line strong cation exchange chromatography and LC–MS/MS

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Abstract

In this study we use replicate 2D-LC–MS/MS analyses of crude membranes from B cells derived from a patient with chronic lymphocytic leukemia (CLL) to examine the protein expression profile of CLL B cells. Protein identifications made by replicate 2D-LC–MS/MS analysis of tryptic peptides from detergent solubilized B cell membrane proteins, as well as replicate LC–MS/MS analysis of single off-line strong cation exchange chromatography (SCX) fractions, were analyzed. We show that despite the variance in SCX, capillary LC, and the data-dependent selection of precursor ions, an overlap of 64% between proteins identified in replicate runs was achieved for this system.

Keywords: CLL; 2D-LC–MS/MS; B cell; Expression profiling; Off-line SCX; Reproducibility

1. Introduction

B cell chronic lymphocytic leukemia (B-CLL) is an adult B cell malignancy affecting both men and women and is the most frequently observed leukemia in Western countries [1]. Clinical diagnosis of CLL typically starts with the observation of large numbers of monoclonal B cells (>5000 μL^{-1}) during a complete blood count with the final diagnosis of CLL typically confirmed using flow cytometry of blood lymphocytes [2]. Recent studies have demonstrated the association of disease state in CLL with specific chromosomal abnormalities [3,4], immunoglobulin (Ig) variable (V) heavy chain (H) gene mutational status (IgV_H) [5,6], and expression of ZAP-70 [7–9]. However, these prognostic tools have yet to be implemented on a widespread basis due to combinations of cost, test availability [10], and validation of their clinical specificity. Therefore, continual refinement is necessary for improved prognostic markers and selected drug targets to better treat CLL. Of interest, CLL B cell expression of ZAP-70 was first identified using gene expression profiling [11], thereby demonstrating the potential of broad screening approaches to identify genes that may permit distinction of disease subgroups. However, new proteomic technologies such as 2D-LC–MS/MS have not been applied to examining the protein content of CLL B cells in an attempt to identify proteins that may be of prognostic value and offer insight into the underlying biology of the malignant B cell.

Abbreviations: 2D-LC–MS/MS, two-dimensional liquid chromatography tandem mass spectrometry; SCX, strong cation exchange; CLL, chronic lymphocytic leukemia; Q-TOF, quadrupole time-of-flight; GPF, gas phase fractionation; IgV_H, immunoglobulin variable heavy chain; RBC, red blood cell; SD, standard deviation; CV, coefficient of variance; M-CLL, chronic lymphocytic leukemia with mutated immunoglobulin variable heavy chain gene; UM-CLL, chronic lymphocytic leukemia with unmutated immunoglobulin variable heavy chain gene

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Examination of the proteins expressed by malignant B cells from CLL patients has been performed for nearly three decades. Much of the protein separation work has been done using 1DE and 2DE [12–15], however, HPLC has also been used [16] with protein detection being done using both autoradiography and standard protein stains. In these early studies proteins were typically identified using Edman sequencing or immunoassay. However, recent advances in ionization methods [17,18] coupled with proteolysis and protein database searching algorithms [19,20] has increased the sensitivity and speed at which proteins can be identified from complex mixtures of proteins through the use of mass spectrometry. Consequently more recent studies examining the protein expression patterns of CLL B cells have incorporated mass spectrometry (for a review of MS applied to hematologic disorders, see Cristea et al. [21]). Examples of current CLL proteomics studies include the 2DE differential work by Voss et al. [22], the 1DE protein profiling work by Boyd et al. [23], and the 2DE study by Cochran et al. [24] comparing CLL B cells derived from patients with and without mutated IgV_H genes.

In this study we evaluate 2D-LC–MS/MS, or MudPIT [25], for identifying proteins from detergent solubilized membranes from CLL B cells as a limited first step in profiling the protein expression of this class of cells. 2D-LC–MS/MS has been shown to be effective for identifying and quantifying proteins expressed in a variety of cells [26], especially when coupled with gas-phase fractionation (GFP) [27–29] and off-line SCX chromatography [30]. Others have also explored the reproducibility of both 2D-LC–MS/MS [31,32] and LC–MS/MS [33] for different biological systems but 2D-LC–MS/MS has yet to be reported as a technology specifically applied to the CLL B cell proteome. Here we show the results of replicate 2D-LC–MS/MS analyses of peptides derived from tryptic digests of CLL B cell preparations. In addition, the analytical variability in the SCX separation, the reverse phase separation, and precursor ion selection, is profiled along with a description of the proteins found from cytoplasm, organelle membranes, and plasma membrane. These findings suggest that proteolysis of CLL B cells, prepared using the methods described, coupled with 2D-LC–MS/MS and protein database searching can provide a broad protein expression profile of CLL B cell membranes and organelle components.

2. Experimental

2.1. CLL B-cell Isolation

Peripheral blood was obtained from an untreated patient diagnosed with classical B-cell CLL using protocols approved by the Mayo Clinic Institutional Review Board. Approximately, 1.1×10^9 peripheral blood mononuclear cells were isolated by Ficoll–Paque gradient centrifugation from 10 mL of blood and then washed once with cold phosphate buffered saline (PBS). Residual erythrocytes were lysed by adding RBC lysis buffer at 37 °C for 10 min followed by two washings with cold PBS. Using two color flow cytometry, ≥95% of the mononuclear cells were positive for both CD5 and CD19.

2.2. Membrane extract

Isolated cells were kept on ice and disrupted in PBS using sonication. Crude membranes were separated from cytosol by ultracentrifugation at $100{,}000 \times g$ for 45 min. The crude membrane pellet was then resuspended in PBS containing 1 M NaCl using sonication to aid in disrupting any non-specific protein interactions with the crude membranes. The suspended crude membranes were spun again at $100{,}000 \times g$ for 45 min. The resulting pellet was rinsed with PBS to remove excess NaCl and frozen at −80 °C. Total protein concentrations were determined in triplicate for cytosol (497 μg/mL) and crude membrane fractions (213 μg/mL) using the BCA method (Pierce, Rockford, IL).

2.3. Solubilization and digestion of crude membrane extract

The membrane pellet was thawed and solubilized in 250 μL of a 100 mM ammonium bicarbonate buffer pH 8.0 containing 6 M urea and 1% octylglucoside using sonication followed by stirring at 4 °C for 30 min. Solubilized proteins were isolated by spinning the sample at $100{,}000 \times g$ for 45 min. Solubilized proteins in the supernatant were reduced with 5 mM DTT for 30 min at 30 °C then alkylated with 15 mM iodoacetamide for 30 min at room temperature in the dark. The sample was diluted three-fold with 100 mM ammonium bicarbonate to reduce the urea concentration to 2 M prior to the addition of trypsin. The sample was proteolyzed with trypsin (Promega, Madison, WI) for 12 h at 37 °C followed by the addition of more trypsin and four more hours of proteolysis all with an enzyme to substrate ratio of 1:10. Following proteolysis the sample was acidified with trifluoroacetic acid (TFA) to a pH ≤ 2 and spun at $14{,}000 \times g$ for 20 min.

2.4. Strong cation exchange chromatography

In order to lower the ionic strength of the digest, tryptic peptides were trapped onto a C_{18} cartridge column (LC Packings) and rinsed with water containing 0.1% (v/v) TFA. A total of 50 μg of protein was loaded onto the cartridge for each replicate. Peptides were eluted with a solution containing 60% (v/v) acetonitrile, 40% (v/v) water, and 0.1% (v/v) TFA. The solution containing the tryptic peptides was then brought to dryness and resuspended in mobile phase A. All SCX chromatography was performed on a Magic 2002 HPLC (Michrom Bioresources, Auburn, CA) using a 'Magic Bullet' polysulfoethyl A cartridge column (28 mm × 1 mm: Michrom Bioresources, Auburn, CA) with a flow rate of

200 μL/min. A gradient elution profile was used starting with 100% mobile phase A (5 mM KH_2PO_4 and 10%, v/v acetonitrile, pH 3.0) then changing to 60% mobile phase B (5 mM KH_2PO_4 and 10%, v/v acetonitrile plus 500 mM KCl, pH 3.0) over 30 min, then ramping to 100% B after 40 min holding for 2 min, then returning to 100% A after 50 min. Fractions were collected every 30 s (100 μL) off the SCX column via an autosampler into a 96-well plate. Plates were covered and stored at 4 °C until analyzed by LC–MS/MS.

2.5. Capillary reverse phase LC

Tryptic peptides contained in each of the fractions analyzed were subjected to capillary reverse-phase LC using a Cap LC system (Waters Corp.). A volume of 5 μL was injected from the SCX fractions and trapped on a Pepmap C_{18} cartridge column (300 μm × 0.5 cm; LC Packings, Amsterdam, NL) via an autosampler. Trapped peptides were washed for 2 min at 10 μL/min with mobile phase A then by using a 10-port valve the Pepmap column was put in-line with the gradient and the reverse-phase column. Peptides were separated using a gradient starting with 95% mobile phase A (98%, v/v water; 1%, v/v *n*-propanol; 1%, v/v acetonitrile; 0.2%, v/v formic acid) and ending with 80% B (80%, v/v acetonitrile; 10%, v/v *n*-propanol; 10%, v/v water; 0.2% formic acid) over the course of a 1 h run. Capillary columns packed in-house for the analysis were packed with TARGA C_{18} (5 μm, 120 Å, Higgins Analytical, Mountain view, CA) in a 75 μm i.d. × 360 μm o.d. fused silica capillary with a length of 5 cm. The flow rate for the reverse-phase analysis was set by splitting the LC pump flow from 12 μL/min to approximately 300 nL/min. The capillary column was connected to a union using a sleeve containing a screen to retain the column particles. The electrospray ionization (ESI) emitter was a 5 cm long piece of fused silica with a 15 μm tip (New Objective, Woburn, MA) connected to the other end of the union and a positive electrospray voltage of approximately 1.2 kV was applied via the source platform.

2.6. MS/MS data

All MS and MS/MS spectra were collected on a Q-TOF API US quadrupole time-of-flight mass spectrometer (Waters Corp.). The instrument was set up to perform a precursor ion scan over one of three *m*/*z* ranges (450–750 *m*/*z*, 700–1000 *m*/*z*, and 950–1250 *m*/*z*). Precursor ions with a positive charge states of 2–4 were selected for MS/MS analysis by the acquisition software and the collision energy voltage ranged from 16 to 65 depending on the charge state and mass of the precursor ion. Raw MS/MS files were converted to .pkl files for database searching using the PeptideAuto function in ProteinLynx. All protein database searches used the human subset of the SwissProt/TREMBL protein database using Matrix Science's Mascot search algorithm performed in-house on a 10-node cluster. The following parameters for the Mascot search were used: carboxyamidomethyl modified cysteine residues; two missed cleavages; oxidation of methionine, histidine, and tryptophan residues; and peptide mass tolerances of ±0.5 Da for both precursor and fragment ions. Protein identifications were checked manually and proteins with a Mascot score of 25 or higher with at least one peptide having a *y* or *b* ion sequence tag of three residues or better were accepted [34].

3. Results

3.1. Off-line SCX chromatography

For the analyses presented here, tryptic peptides were first separated by off-line SCX chromatography with fractions being collected directly into 96-well plates. A total of three different SCX runs were performed on the same digest. Fractions were then subjected to three different capillary RP-LC–MS/MS runs corresponding to three different GPF precursor ion scan ranges. Fig. 1 shows the results from the three SCX runs where an arbitrarily selected peptide was followed in the three different runs. The figure first shows the TIC for each of the three replicate SCX runs that contained the peptide in the GPF range of 950–1250 *m*/*z*, Fraction 6, SCX Run 1 (top), Fraction 11, SCX Run 2 (middle), and Fraction 10, SCX Run 3 (bottom). The selected ion chromatograms shown in Fig. 1 were used to determine the reverse phase elution time for this peptide in each of the three runs. A retention time S.D. of 1.3 min with a CV = 4.4 was calculated for this particular peptide from the replicate runs.

The total number of MS/MS spectra collected for these SCX fractions were very similar with the following values; Fraction 6 = 177, Fraction 11 = 162, Fraction 10 = 189, CV = 7.7, suggesting that similar numbers of precursor ions were present in each fraction capable of triggering data dependent MS/MS acquisition. The total number of proteins identified for each fraction with acceptable Mowse scores were also very similar with the following values; Fraction 6 = 40, Fraction 11 = 42, Fraction 10 = 41, CV = 2.4. The mean overlap in the number of proteins common to each run was 37% found by taking the mean from the number of proteins common to all three runs (15) divided by the number of proteins found in each SCX fraction. The low overlap in protein identifications between these three runs is most likely attributable to the differences in the SCX retention times for peptides in Runs 1–3. Run 1 was acquired 3 weeks before Runs 2 and 3 and produced an elution profile where the same peptides eluted roughly 2 min earlier than in Runs 2 and 3 (data not shown). This shift could be caused by a number of different factors including column conditioning prior to injection, however, as will be shown later in the text, retention time shifts in the SCX dimension do not appear to have a major impact on the overlap in protein identifications between 2D-LC–MS/MS analyses.

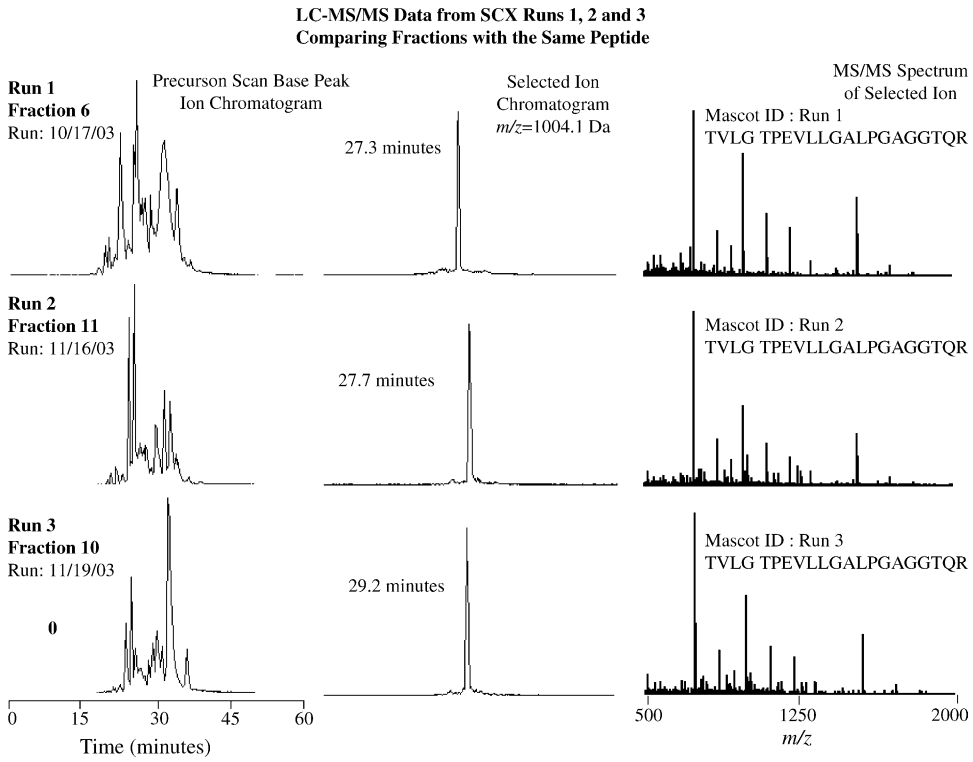

Fig. 1. Comparison of SCX fractions containing the same peptide from replicate 2D-LC–MS/MS runs. A tryptic peptide was arbitrarily selected as a marker to examine the reproducibility between SCX Runs 1–3. The figure shows that despite differences in SCX fraction number and reverse-phase HPLC retention time, the peptide was selected in the precursor ion scan and subjected to MS/MS giving rise to the same Mascot identification in each run.

3.2. Replicate injections of the same off-line SCX fractions

To evaluate the reproducibility that could be expected with our system in the reverse phase dimension, we performed LC–MS/MS on the same SCX fraction. Fig. 2 shows the results from SCX fraction 12 Run 2, GPF range 950–1250, injected three times. Retention times are listed for peaks containing the same peptide, as identified by a Mascot search, at the beginning of the run (retention time standard devia-

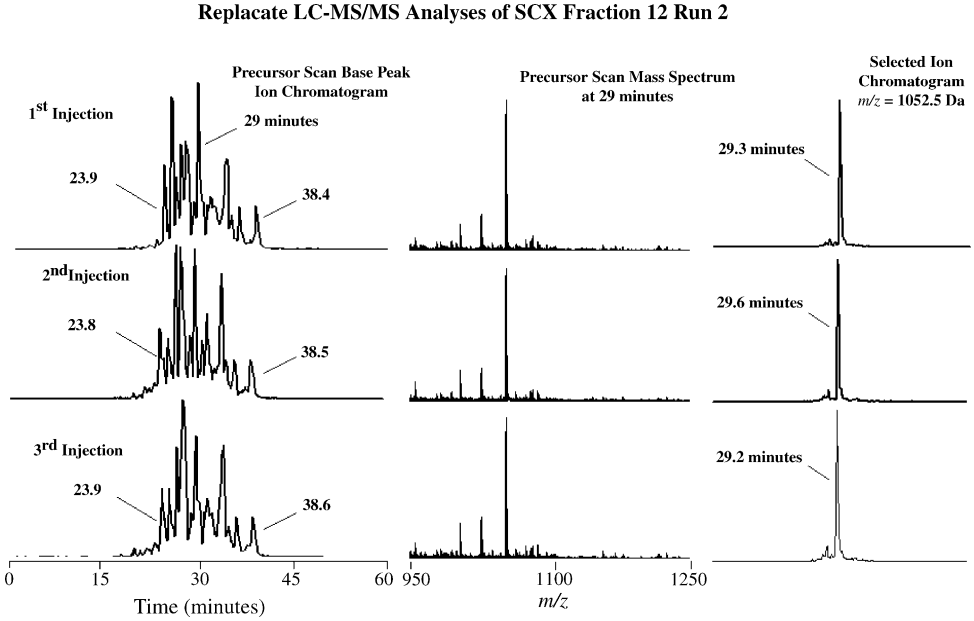

Fig. 2. Comparison of replicate LC–MS/MS analyses of the same SCX fraction. SCX Fraction 12 (Run 2) was arbitrarily selected to be examined three times by LC–MS/MS. The figure illustrates the reproducibility in retention times and mass spectra collected between each of the replicate runs from Fraction 12. Fraction 12 (Run 3) was also selected for triplicate analysis and results from both runs are described in the text.

tion, S.D. = 1.8 s, CV = 0.13) and at the end of the run (retention time S.D. = 4.8 s, CV = 0.21). The middle of Fig. 2 shows three mass spectra from a precursor ion scan at roughly 30 min and on the right are three selected ion chromatograms for the base peak ion at $m/z = 1052.5$ with a retention time S.D. = 12 s and CV = 0.58. This peptide was determined to be the best match by a Mascot search for the tryptic peptide GMSLNLEPDNVGVVVFGNDK containing residues 104–123 from the alpha chain of mitochondrial ATP synthase.

Triplicate analysis of a single SCX fraction was also performed on SCX fraction 12 from Run 3, GPF range 950–1250. Mascot search results of MS/MS data from triplicate injections of SCX Fraction 12 from Runs 2 and 3 showed that the number of proteins with Mowse scores above an acceptable value, and the number of MS/MS scans acquired, were similar for each of the three runs. For example, the number of MS/MS spectra acquired for SCX Fraction 12 from Run 2 were 177, 179, and 182 with a CV = 1.4, while the number of MS/MS spectra acquired for SCX Fraction 12 from Run 3 was 220, 221, and 224 with a CV = 0.94. These values suggest that the actual protein identifications should be very similar if the same peptides were selected for MS/MS from each run. However, the calculated mean overlap as a percentage was found to be 64% for triplicate injections of SCX fraction 12 from Run 2, and 59% for triplicate injections of SCX Fraction 12 from Run 3. As a measure of reproducibility, these overlap values are poor as compared to retention time reproducibility observed in these analyses. This reiterates the fact that even with good reverse-phase capillary chromatography the timing of the data dependent acquisition, along with the variability in MS/MS spectral interpretation, ultimately dictates reproducibility in protein identification [27,29].

3.3. CLL B cell proteins identified from merged MS/MS files

The Venn diagram in Fig. 3 illustrates the similarities and differences in the proteins identified in the three 2D-LC–MS/MS runs. As the figure shows the number of proteins found to be the same in each run was 318 while the total number of proteins identified per run after manual confirmation was 464 for Run 1, 519 for Run 2, and 500 for Run 3. The calculated percent overlap from the three analyses was 64%, very similar to what was observed for replicate LC–MS/MS analysis of the same SCX fractions, fractions 12 from Run 2 (64%) and Run 3 (59%). This result demonstrates further how protein identifications from replicate 2D-LC–MS/MS analyses depend primarily on the precursor ion selection process in the MS/MS portion of the experiment.

3.4. Cellular locations of CLL B cell proteins identified from 2D-LC–MS/MS

The subcellular locations of the proteins identified from the digest of CLL B cell crude membranes are shown in Fig. 4. The subcellular locations were found in the SwissProt, NCBI,

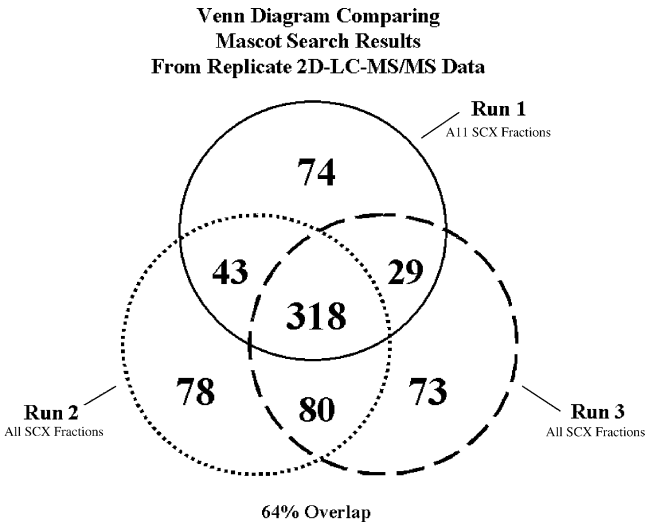

Fig. 3. Venn diagram comparing the protein identifications from 2D-LC–MS/MS Runs 1–3. The diagram illustrates similarities and differences in the proteins identified in each of the three runs by manually confirmed Mascot searches of MS/MS data. The percent overlap observed between the total number of proteins common to all runs and the mean total number of proteins identified from all three runs was found to be 64%.

and iProClass databases using the Gene Ontology (GO) Consortium [35] as a reference. The subcellular locations of the proteins identified in the three runs were very similar as indicated by the overlap value of 64% and the proteins that were different between runs (74 in Run 1, 78 in Run 2, and 73 in Run 3) did not have an impact on the numbers of proteins in each category. For example, when the numbers of proteins present in cytoplasm, mitochondria, membrane, and nucleus are normalized to the total number of proteins in

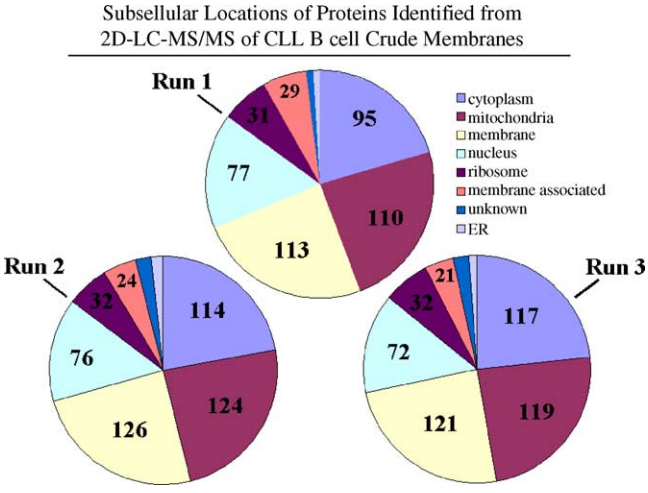

Fig. 4. Charts showing the subcellular locations for proteins identified from Mascot searches of the SwissProt database for replicate 2D-LC–MS/MS analyses. The charts show the diverse distribution of proteins identified from the crude membrane extract of CLL B cells. The similarities in the distribution values from each chart demonstrate that different proteins identified in each run did not significantly change the distributions between each run.

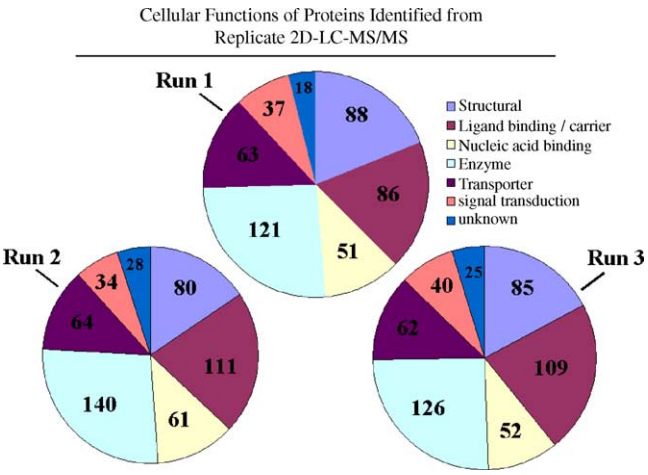

Fig. 5. Charts showing the cellular functions for proteins identified from Mascot searches of the SwissProt database for replicate 2D-LC–MS/MS data. The values shown for each of the cellular functions reflect the findings in Fig. 4 and shows the broad range of functionalities for proteins identified from the crude membrane extract of CLL B cells.

each run, the CV values observed ranged from 0.22 to 7.8. These values suggest that no tryptic peptides were preferentially selected in one run over the next. Fig. 5 shows a set of pie charts illustrating the distribution of known functions for the proteins identified in the three runs developed using the database resources listed above and reiterate the findings shown in Fig. 4. The results displayed in Figs. 4 and 5 were encouraging since the goal of using a crude membrane preparation was to save time and yield a broad expression profile of proteins, not just proteins from a single subcellular location. A single 2D-LC–MS/MS analysis was performed on a tryptic digest of the cytosol fraction of the preparation to evaluate its protein composition as compared to the crude membrane fraction. A total of 345 proteins were identified from the cytosol fraction, 121 of which were found to overlap with those identified in the crude membrane digest. It was found that 25% of the cytosol fraction proteins identified were membrane proteins whereas 20% of the proteins identified in the crude membrane preparation were cytoplasmic proteins.

4. Discussion

The decision to use a routine, easily automated, sample preparation procedure for separating membranes (plasma membranes, and organelle membranes) from cytosol was based on anticipated needs for analyzing CLL patient samples as part of future efforts to characterize CLL B cell protein expression on a quantitative and qualitative level. While not as stringent as density gradient centrifugation for isolating pure plasma membranes, sonication coupled with ultracentrifugation is a robust approach to simplifying the complexity of a cell extract giving a broad representation of membrane proteins. This is illustrated by western blot and 1DE data on equal protein loads of crude membrane extracts done in triplicate from the same starting cells which suggest that the extraction process is reproducible (data not shown). Table 1 compares previously reported proteins identified from 1DE separated plasma membranes and 2DE separated whole cell extracts with the 2D-LC–MS/MS data presented in this study. Table 1 shows that the results from the crude membrane extract can give complementary data as well as equivalent results for protein identifications from CLL B cell preparations. To facilitate future comparisons with CLL B cell protein expression data, a list of the proteins we identified from a merged .pkl file that included the MS/MS data from all three 2D-LC–MS/MS runs of crude membrane digests are included in Supplementary material. The proteins listed are sorted by their respective Mascot Mowse score and include the following information; (1) SwissProt ID, (2) SwissProt accession number, (3) Protein score, (4) the peptide(s) sequence used for the identification (5) the run(s) where the peptide was observed (6) the highest score for each sequence, (7) the number of MS/MS spectra assigned to each sequence, and (8) the number of MS/MS spectra assigned to an oxidized methionine residue. While it is difficult to compare the relative abundance of proteins identified from silver stained 1DE and 2DE gels with the relative abundances of proteins identified by 2D-LC–MS/MS, the data presented here can be used to examine the most prevalent tryptic peptides selected for MS/MS using the crude membrane preparation of CLL B cells.

Table 1
Comparison of CLL B cell proteins identified by 1DE, 2DE and 2D-LC–MS/MS

	1DE	2D-LC–MS/MS
CD markers [23]	CD5, CD19, CD20, CD22, CD23 CD11a/b/c, CD29, CD41, CD49d/c, CD51, CD53, CD72, CD73, CD166	CD5, CD20, CD22, CD23 CD37, CD42, CD44, CD45, CD81
Total number of proteins identified	500 from plasma membranes	695 crude membranes[a] 346 cytosol
	2DE	**2D-LC–MS/MS**
Genotype and protein expression [22]	17 differentially expressed proteins ID'd	12 of 17 identified
UM-CLL vs. M-CLL [24]	57 survey and differentially expressed proteins ID'd (approximately 800 spots estimated per gel)	46 of 57 identified

Proteins identified in 1DE, 2DE and 2D-LC–MS/MS. The table shows the capacity of 2D-LC–MS/MS to give concurrent and complimentary proteins identifications including CD markers specific to CLL B cells.

5. Concluding remarks

In this study we evaluated replicate 2D-LC–MS/MS analyses coupled with protein database searching as a means of examining the protein expression profile of CLL B cells from a single patient. The overlap observed for the protein identifications from the three separate runs using the same digest resulted in a protein identification overlap of 64%. A list of proteins manually confirmed as passing specific confidence criteria are provided in Supplementary material. While not inclusive, this list serves as a start for future reference in regards to such topics as, the types of proteins found using sonication and ultracentrifugation in sample preparations of CLL B cells, the reproducibility expected for 2D-LC–MS/MS utilizing the instrumentation and software tools described for this cell type, high abundance peptides that should be excluded to help increase protein coverage, and as a comparison to other proteomic and genomic data for CLL B cells. It is worth noting that the crude membrane preparation methodology coupled with 2D-LC–MS/MS can be highly automated. After membrane isolation and digestion the analyses performed for this study were automated from the point of injection onto the off-line SCX system until submission to a Mascot search.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2005.01.021.

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