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Combined Chemical and Enzymatic Stable Isotope Labeling for Quantitative Profiling of Detergent-insoluble Membrane Proteins Isolated Using Triton X-100 and Brij-96

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Abstract

Effective quantitative profiling of detergent-insoluble membrane proteins using high-throughput mass spectrometry (MS)-based proteomics would allow a better understanding of physiological and pathological processes that take place at the cell surface. To increase the coverage of proteins present in detergent-resistant membrane microdomains (DRMMs), a combination of $^{16}O/^{18}O$ and isotope coded affinity tags (ICAT) labeling was used in a comparative analysis of detergent-insoluble membrane proteins isolated from rat basophilic leukemia cells (RBL-2H3), with either Triton X-100 or Brij-96. The analysis resulted in the quantification of 738 unique proteins from Triton X-100 and Brij-96 isolated DRMMs, significantly exceeding the number of proteins quantified from either single labeling technique. Twenty-five non-cysteine-containing proteins were quantified, as well as 32 cysteine-containing proteins that would have been missed if either $^{16}O/^{18}O$ or ICAT labeling had been used exclusively, which illustrate better proteome coverage and enhanced ability to quantitate. The comparative analysis revealed that proteins were more readily extracted using Triton X-100 than Brij-96; however, Triton X-100 also extracted larger quantities of non-DRMMs-associated proteins. This result confirms previous, targeted studies suggesting that DRMMs isolated using Triton X-100 and Brij-96 differ in their protein content.

Keywords

Quantitative proteomics; combined $^{16}O/^{18}O$ and ICAT stable isotopic labeling; Triton X-100 and Brij-96 detergent-insoluble membrane proteins

Introduction

Current advancements in the field of mass spectrometry (MS)-based quantitative proteomics offer the opportunity to elucidate key regulators and/or effectors that are relevant to physiological or pathological cellular processes. For this reason, quantitative profiling of proteins from cells or organisms exposed to various stimuli has been increasingly employed in proteomic research [1–5]. Recently, an array of MS-based methods for protein quantitation,

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employing different stable isotopic labeling techniques, has been developed as an alternative to two-dimensional gel electrophoresis-based comparative proteomics [6–11]. Depending on the mode of stable isotopic labeling, the most frequently used methods can be divided into three groups: a) metabolic labeling, b) chemical labeling, and c) enzyme-catalyzed labeling. Metabolic labeling of cultured cells or organisms uses isotopically enriched medium or diet to incorporate specific heavy isotopic atoms within the entire proteome. Metabolic labeling is best illustrated by the stable isotope labeling by amino acids in cell culture (SILAC) [9]. Chemical stable isotope labeling is conducted by chemically modifying the proteome after it has been extracted from the sample of interest. It is exemplified by the isotope-coded affinity tag (ICAT) method [7, 12] and its variant that utilizes isobaric amine specific tags named iTRAQ [11, 13]. Enzyme-catalyzed labeling is carried out during the enzymatic digestion of an extracted proteome sample in the presence of $H_2{}^{16}O$ or $H_2{}^{18}O$ [6, 8, 14].

The objectives of this work were to develop a quantitative approach that would allow more effective profiling of complex membrane protein mixtures and to investigate the effect of two distinct non-ionic detergents on the global level of protein enrichment from differently isolated DRMMs. It is widely accepted that DRMMs isolated from low-density sucrose-gradient fractions are regarded as lipid rafts and contain membrane proteins that are often implicated in signal transduction [15]. The most common methods of isolating lipid rafts involve the use of detergents while particular investigations were carried out using gel-based[16, 17] or solution based proteomic approaches[18]. The choice of detergent may, however, influence the abundance of any particular protein that is isolated within the extracted lipid raft, due to the differential solubility of membrane proteins in detergents [19, 20]. In this study, DRMMs were differently isolated from rat basophilic leukaemia (RBL-2H3) cells, using either Triton X-100 or Brij-96. The DRMMs were simultaneously labeled using a combination of $^{16}O/^{18}O$ and ICAT stable isotope labeling and analyzed using nanoflow reverse-phased liquid chromatography coupled on-line with data-dependent tandem MS (RPLC-MS/MS). The RBL-2H3 cell line was selected because it has been shown that these cells express high levels of well-characterized lipid raft marker proteins and are a reliable model to study DRMMs [21–23]. While these two labeling techniques have previously been used individually for quantitative profiling of complex protein mixtures [12, 24], this is the first report of their adaptation for simultaneous application on a single membrane protein sample.

Experimental procedures

Materials

Ammonium bicarbonate, dibasic sodium phosphate, monobasic sodium phosphate, phenylmethanesulfonyl fluoride (PMSF), pepstatin A, leupeptin, and 95% v/v $H_2{}^{18}O$ were purchased from Sigma (St. Louis, MO). The light $^{12}C_9$-ICAT reagent and the heavy $^{13}C_9$-ICAT isotopic versions were purchased from ABI, (Framingham, MA). Sequencing grade trypsin was obtained from Promega (Madison, WI). Trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Fluka (Milwaukee, WI). HPLC grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from EM Science (Darmstadt, Germany). UltraLink immobilized monomeric avidin, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), ImmunoPure D-biotin, bicinchonic acid (BCA) protein assay reagent kit, and Excellulose size exclusion chromatography columns were purchased from Pierce (Rockford, IL). Triton X-100 was from Roche Diagnostic (Laval, QC, Canada) and Brij-96 was obtained from Fluka (Oakville, ON, Canada). Water was purified by a Barnstead Nanopure system (Dubuque, IA). The rat basophilic leukemia cell line (RBK-2H3) was obtained from A.T.C.C. (Manassas, VA).

Isolation of DRMMs

Cells, grown as monolayers in minimal essential medium, were washed in PBS (pH 7.4) and the detergent-resistant membrane fractions were isolated using a method adapted from and described by Prinetti *et al* [25]. Briefly, cells were lysed on ice for 30 min using a buffer consisting of either 0.5% (w/v) Brij-96 in 25 mM Tris/HCl and 140 mM NaCl (pH 7.5) or 1% (w/v) Triton X-100 in 25 mM Tris/HCl and 140 mM NaCl (pH 8.0) along with 1 mM PMSF, 1 mM Na_3VO_4 and a cocktail of protease inhibitors. Lysate was centrifuged at 10,000 *g* for 5 min to obtain post-nuclear lysate that was ultracentrifuged at 400,000 *g* for 4 hours (Beckman Coulter, VTi 65.2 rotor) using discontinuous sucrose gradients, 40%, 30% and 5% respectively. A total of 13 fractions of 0.4 mL were collected from the top of the gradient. Low-density raft-containing fractions (2–7) were pooled (protein content determined by BCA assay), pelleted at 400,000 *g* for 1 hour and stored at −80 °C for subsequent proteomic analysis. All experimental procedures were performed at 4 °C.

SDS-PAGE and Western immunoblot analysis

Sucrose gradient fractions (0.4 ml each) were collected from Triton X-100 (control sample) and Brij-96 (compared sample), respectively. Representative amounts of each fraction were analyzed by SDS-PAGE on a 10% acrylamide gel [26]. All collected sucrose gradient fractions, starting from the top of the gradient, were immunoblotted, using primary antibodies (i.e., anti-Yes, anti-CD71) to identify fractions containing lipid-raft microdomains (data not shown), while low-density DRMMs fractions were immunoblotted using anti-Lyn primary antibody to determine the level of this lipid-raft marker within the Triton X-100 and Brij-96 fractions, respectively. Briefly, resolved proteins were transferred on to a nitrocellulose membrane blocked in 5% (w/v) milk in TBS-Tween buffer (pH 7.5). Subsequently, membranes were incubated with the primary antibody of choice and further incubated by HRP-conjugated gout anti-mouse antibody. Specific interactions were revealed by an ECL® (enhanced chemiluminesence) detection system (Amersham Biosciences, Baie d'Urfé, QC, Canada).

Solubilization and combined $^{16}O/^{18}O$ and ICAT labeling of proteins from DRMMs

A schematic of labeling procedure is shown in Fig. 1. Equal amounts of DRMMs (100 μg each) were sequentially labeled using ICAT and $^{16}O/^{18}O$ stable isotope labeling regents. A Triton X-100 isolated protein sample (control) was labeled using light ($^{12}C_9$-ICAT and $H_2{}^{16}O$) reagents, while the Brij-96 isolated compared protein sample was labeled with heavy ($^{13}C_9$-ICAT and $H_2{}^{18}O$) reagents. Initially, each detergent-resistant pellet was resuspended in 1.5 ml of 25 mM NH_4HCO_3 (pH 7.9), pelleted by ultracentrifugation (100,000 g for 1 h) and supernatant discarded. The same washing procedure was repeated two times to remove detergent residues and carry out a buffer exchange. Following the last washing, each pellet was separately solubilized, using intermittent sonication and vortexing in 0.2 ml of CH_3OH (60% v/v) buffered with 50 mM NH_4HCO_3, pH 7.9 as previously described [27]. Once solubilized, both fractions were separately labeled using ICAT reagents as previously described [28]. Each sample was chemically reduced using 1mM TCEP-HCl (final concentration) at 37 °C for 30 min and transferred to vials containing either light $^{12}C_9$-ICAT or heavy $^{13}C_9$-ICAT reagents solubilized in 20 μl of HPLC grade acetonitrile. Labeling reactions were performed separately by incubation at 37 °C for 2 hours, followed by desalting, using two Excellulose-packed, size-exclusion columns equilibrated with 60% (v/v) buffered methanol. The desalted light and heavy ICAT-labeled protein samples were digested separately overnight using the same solubilizing buffer and 1:20 (w/w) trypsin/protein ratio. After proteolysis, the heavy ($^{13}C_9$-ICAT) labeled digestate (compared Brij-96 isolated sample) was lyophilized and C-termini of heavy $^{13}C_9$-ICAT labeled biotin-tagged peptides as well as C-termini of unlabeled biotin-free non-cysteine-containing peptides were labeled by heavy $^{18}O_2$ tag. The ^{18}O labeling was carried out overnight employing trypsin

catalyzed ^{18}O exchange in 100 μL of 20% CH_3OH/80% 25 mM NH_4HCO_3, pH 7.9 prepared in $H_2{}^{18}O$, using a 1:20 trypsin/protein ratio as previously described [24]. The identical procedure was carried out on the light-labeled $^{12}C_9$-ICAT digestate (Triton X-100 isolated control sample) using 20% CH_3OH/80% 25 mM NH_4HCO_3, pH 7.9 prepared in $H_2{}^{16}O$. The exchange reactions were quenched by boiling the samples for 3 min in a water bath and cooling them to room temperature, followed by addition of PMSF (1 mM final concentration). The sample for comparison, Brij-96 isolated DRMMs, was comprised of a heavy isotope-labeled peptide pool, containing a mixture of $^{18}O_2$-labeled and dually $^{18}O_2{}^{13}C_9$-ICAT-labeled peptides, while the control sample, Triton X-100 isolated DRMMs, was comprised of a light isotope-labeled peptide pool containing a mixture of $^{16}O_2$-labeled and dually $^{16}O_2{}^{12}C_9$-ICAT-labeled peptides. Both peptide pools were combined prior to avidin affinity chromatography, as shown in Fig. 1. Accordingly, the combined peptide mixture contained biotin-free differentially $^{16}O/^{18}O$-labeled peptides (i.e., $^{16}O_2$ and $^{18}O_2$-labeled non-cysteine containing peptides) and biotin-tagged dually $^{16}O/^{18}O$-ICAT-labeled peptides (i.e., $^{18}O_2{}^{13}C_9$-ICAT and $^{16}O_2{}^{12}C_9$-ICAT-labeled cysteine-containing peptides) ready for avidin-affinity chromatographic separation.

Avidin-affinity chromatographic separation of differentially biotin-free $^{16}O/^{18}O$-labeled peptides from biotin-tagged $^{16}O/^{18}O$-ICAT dually labeled peptides

An UltraLink immobilized monomeric avidin column (0.4 ml bed volume) was slurry-packed in a glass Pasteur pipette, equilibrated with 2× PBS, pH 7.2, and blocked using 2 mM D-biotin in 2× PBS, pH 7.2. The biotin was removed from the reversible binding sites following the manufacturer's instructions, and the column was equilibrated using 2× PBS, pH 7.2, followed by two washes using 25 mM NH_4HCO_3. The combined peptide mixture was loaded on the avidin column and the unbound (flow-through) biotin-free fraction containing $^{16}O_2/^{18}O_2$-labeled peptides was collected. After a 20 min incubation at ambient temperature, the column was washed with 25 mM NH_4HCO_3 and the eluate was collected. These two collections were combined as biotin-free $^{16}O_2/^{18}O_2$-labeled fraction A and lyophilized to dryness. The column was then washed with 5 bed volumes each of 2× PBS, pH 7.2 and 1× PBS pH 7.2. The $^{16}O_2{}^{12}C_9$-ICAT /$^{18}O_2{}^{13}C_9$-ICAT-labeled biotin-tagged peptide fraction B was eluted using 1.5 mL of 30% (v/v) CH_3CN, 0.4% (v/v) FA. The biotin tags were then cleaved from the modified peptides according to the manufacturer's instructions, and the resulting peptide mixture was lyophilized to dryness.

SCX-LC fractionation of differentially biotin-free $^{16}O/^{18}O$-labeled and biotin-tagged $^{16}O/^{18}O$-ICAT dually labeled peptide fractions

The biotin-free $^{18}O/^{16}O$-labeled peptide fraction was reconstituted in 100 μl of 45% (v/v) CH_3CN containing 0.1% (v/v) FA immediately prior to SCX-LC and resolved into 10 fractions using a microcapillary HPLC system (Model 1100, Agilent Technologies Inc., Palo Alto, CA). Mobile phase A was 45% (v/v) CH_3CN and mobile phase B was 45% (v/v) CH_3CN containing 0.5 M ammonium formate, pH 3. Peptide fractions were eluted with an ammonium formate/multistep gradient at a flow rate of 200 μL/min as follows: 1% B/0–2 min, 10% B/62 min, 62% B/82 min, 100% B/85 min. The biotin-tagged, dually $^{16}O/^{18}O$-ICAT-labeled, cysteine-containing peptide fraction was reconstituted in 100 μl of 45% (v/v) CH_3CN containing 0.1% (v/v) FA immediately prior to SCX-LC and resolved into four fractions using the same procedure. The resulting SCX-LC fractions of both biotin-free and biotin-tagged peptide samples were lyophilized to dryness and reconstituted in 0.1% formic acid immediately before μLC-ESI-MS-MS/MS analysis.

Reversed phase nanoflow liquid chromatography electrospray tandem mass spectrometry

Reversed-phase LC separations were carried out using a 75 μm i.d. × 10 cm-long fused silica capillary (Polymicro Technologies Inc., Phoenix, AZ) column with a flame-pulled tip

(~5–7 μm orifice). The column was slurry-packed in-house with 3 μm, 300 Å pore size C-18 stationary phase (Vydac, Hercules, CA), using a slurry-packing pump (Model 1666, Alltech Associates, Deerfield, IL). After injecting 6 μl of sample, the column was washed for 30 min with 98% mobile phase A (0.1% v/v FA) and peptides were eluted using a linear step gradient from 2 to 60% mobile phase B (0.1% FA in CH_3CN) over 100 min and 60–98% mobile phase B over 20 min at a constant flow rate of 0.25 μL/min. The column was washed for 20 min with 98% mobile phase B and re-equilibrated with 2% mobile phase B for 30 min prior to subsequent sample loading. The reversed-phase column was coupled to either a quadrupole ion trap mass spectrometer (LCQ Deca XP, ThermoElectron, San Jose, CA) or to a hybrid linear ion trap (LIT)-Fourier transform ion cyclotron resonance (FTICR) MS (LTQ-FT, ThermoElectron, San Jose, CA) for global proteomic analysis DRMM protein, differentially extracted using Triton X-100 and Brij-96, respectively. In both analyses a nano-electrospray ionization source was employed, applying a potential of 1.7 kV, and capillary temperature of 160 °C. The mass spectrometers were operated in a data-dependant mode. The most abundant peptide molecular ions detected by MS were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 36%. Dynamic exclusion was employed to avoid redundant acquisition of precursor ions previously selected for MS/MS.

Data analysis

The CID spectra were analyzed using SEQUEST, on a Beowulf 18-node parallel virtual machine cluster computer (ThermoElectron), against a non-redundant rat proteome database (http://www.ebi.ac.uk/integr8/EBI-Integr8-HomePage.do (release date 12/08/03). Only peptides possessing tryptic termini (allowing for up to two internal missed cleavages) possessing delta-correlation scores (ΔCn) ≥0.08 and charge state-dependent cross correlation (Xcorr) criteria as follows were considered as legitimate identifications: ≥1.9 for $[M+H]^{1+}$ peptides, ≥2.2 for $[M+2H]^{2+}$ peptides, and ≥2.9 for $[M+3H]^{3+}$ peptides. A dynamic modification of 4.008 was set on the carboxy-terminal of each $^{18}O_2$-labeled peptide, representing a mass difference for differentially $^{16}O/^{18}O$-labeled peptides. For dually $^{18}O/^{16}O$-ICAT-labeled peptides, a dynamic modification of 4.008 was set on the carboxyl-terminus, along with static modification of 227.127 for light $^{12}C_9$-ICAT-labeled peptides and dynamic modification of 9.03 for heavy $^{13}C_9$-ICAT-labeled peptides. Relative abundances of identified $^{18}O/^{16}O$-labeled peptides and dually $^{18}O/^{16}O$-ICAT-labeled peptides were quantified respectively, using XPRESS (ThermoElectron) software. The abundance ratios (AR) of the peptides and proteins isolated from the RBK-2H3 cells are reported as heavy-to-light (i.e., Brij-96 extracted value divided by the Triton X-100 extracted value). The mapping of α-helical transmembrane domains for selected integral membrane proteins was performed using the transmembrane hidden Markov model (TMHMM) software algorithm that can be found at (http://www.cbs.dtu.dk/services/TMHMM).

Results and discussion

The utility of combined $^{16}O/^{18}O$ and ICAT stable isotope labeling

To prove the principle that membrane proteins from a complex protein mixture can be dually $^{16}O/^{18}O$-ICAT-tagged using ICAT labeling at the protein level and $^{16}O/^{18}O$ labeling at the peptide level, two equivalent amounts of Triton X-100 isolated DRMMs from RBL-2H3 cells were dually $^{16}O/^{18}O$-ICAT-labeled and analyzed using quadrupole ion trap nanoflowRPLC-MS-MS/MS. A mass spectrum of doubly charged peptide pair, exhibiting a $\Delta m/z$ of 6.52, illustrates the detection of light and heavy isotopomer and confirms the successful labeling by both ICAT and $^{16}O/^{18}O$ stable isotope reagents (Fig. 2A). The tandem mass spectra of each isotopomeric peptide molecular ion displayed in Fig. 2A are

shown in Figs. 2B and 2C. These spectra illustrate the identification of the light $^{16}O_2/^{12}C_9$-ICAT dually labeled (R.C*SSILLHGK.E) and the heavy $^{18}O_2/^{13}C_9$-ICAT dually labeled (R.C$^{\#}$SSILLHGK^.E) peptides originating from sodium/potassium-transporting ATPase, a well characterized plasma membrane protein marker [29]. The abundance ratios (AR) for two peptides extracted and quantitatively measured from this integral membrane protein using the dual $^{16}O/^{18}O$-ICAT labeling method are shown in Table 1. The AR was close to unity for both peptides, as expected.

Nanoflow-RPLC-LIT-FTICR-MS analysis

In the present investigation, DRMMs isolated from RBL-2H3 cells, using either Triton X-100 or Brij-96, were quantitatively profiled employing the combined $^{16}O/^{18}O$ and ICAT stable isotopic labeling scheme depicted in Fig. 1. The SCX peptide fractions were analyzed using nanoflowRPLC coupled to a hybrid LIT-FTICR mass spectrometer operating in data-dependent MS/MS mode. Comparative profiling of differently isolated DRMMs using Triton X-100 (control sample) and Brij-96 (compared sample) resulted in quantitation of 7,105 fully tryptic peptides corresponding to 738 uniquely quantified proteins (Supplementary Tables 1–4 available online). The Venn diagram in Fig. 3 shows that 609 proteins were exclusively quantified from $^{16}O/^{18}O$-labeled peptide fraction A, and 32 exclusively from dually $^{16}O/^{18}O$-ICAT-labeled peptide fraction B, while 97 proteins (overlap) were quantified from both peptide fractions AB (Supplementary Table 5 available online). The identification of a number of well-characterized raft marker proteins (i.e., caveolin, flotillin, Thy-1, Lyn) along with quantitation of TEC-21, which is a recognized RBL-2H3 cell-specific raft marker[22], indicate the effective isolation of DRMMs.

Since cysteine-containing peptides represent only a small percentage of a tryptically digested proteome it could be anticipated that the dually $^{16}O/^{18}O$-ICAT-labeled peptide fraction B would contain a considerably smaller number of quantified proteins. Indeed, only 129 proteins were quantified within this fraction. However, 32 proteins were exclusively quantified within this dually $^{16}O/^{18}O$-ICAT-labeled fraction (Supplementary Table 5) and were not observed within biotin-free $^{16}O/^{18}O$-labeled peptide fraction A. These biotin-tagged, $^{16}O/^{18}O$-ICAT dually labeled peptides were captured and eluted from an avidin affinity column, resulting in reduction of complexity of the peptide sample that has been analyzed by RPLC-MS/MS. This reduction in complexity increases the ability of the mass spectrometry to select and identify lower abundant peptides that might otherwise be missed in the relatively more complex mixture of peptides that is exclusively $^{16}O/^{18}O$-labeled. The mass spectrum shown in Fig. 4A illustrates the detection of a peptide pair representing the light and heavy isotopomers from dually $^{16}O/^{18}O$-ICAT-labeled protein. The fragment ion series shown in Figs. 4B–C show the corresponding MS/MS spectra that identify the light Triton X-100 isolated (Figure 4B) and the heavy Brij-96 isolated (Figure 4C) isotopomer of K.YNFFCQGTR.S peptide originating from fatty acid elongase. The extracted ion chromatograms of the same peptides (Fig. 5) reflect their corresponding abundances in the Triton X-100 (Figure 4A) and Brij-96 (Fig. 4B) isolated fractions, and show a calculated heavy-to-light ratio of 0.31, indicating that this protein is more readily isolated using Triton X-100. The fatty acid elongase has been previously characterized as a lower abundance membrane enzyme involved in the biosynthesis of C18 fatty acids [30]. We hypothesize that avidin-facilitated enrichment of cysteine-containing peptides from the biotin-tagged $^{16}O/^{18}O$-ICAT-labeled peptide fraction B allowed identification and quantitation of these proteins, while corresponding non-cysteine containing peptides from the $^{16}O/^{18}O$-labeled fraction A were not detected due to the limited dynamic range afforded in the analysis of such complex peptide mixtures. A sub-set of cysteine-containing membrane proteins, exclusively quantified from dually $^{16}O/^{18}O$-ICAT-labeled peptide fraction B, is shown in Table 3.

Twenty-five (4.1%) of the 609 proteins exclusively identified from the $^{16}O/^{18}O$-labeled peptide fraction A contained no cysteinyl residues in their sequence and therefore would be impossible to quantitate using ICAT labeling (Supplementary Table 6). A subset of non-cysteine-containing integral membrane proteins quantified from this fraction that would be missed if ICAT labeling was exclusively used is shown in Table 4. In addition, the identification of proteins possessing unfavorable distribution of cysteinyl residues that preclude generation of tryptic peptides suitable to a typical *m/z* range of contemporary MS would have been missed as well. This situation is exemplified by the protein DAD-1 (defender against cell death 1), shown in Table 2. DAD-1 (P61805) is an integral membrane protein essential for survival of cultured cells [31]. This 112 amino acid residue protein, (SASVVSVIS**R**FLEEYLSSTPQ**R**L**K**LLDAYLLYILLTGALQFGY**C**L LVGTFPFNSFLSGFISCVGSFILAV**C**L**R**IQINPQN**K**ADFQGISPE**R**AFADFLFASTIL HLVVMNFVG) contains two cysteinyl residues (red font). Tryptic digestion of this protein results in both cysteinyl residues being contained within a 49-residue peptide with a molecular mass of 5355 Da. Unless the peptide was observed in a 3^+ or greater ionization state, it would not be detected by the mass spectrometer from biotin-tagged fraction using the parameters employed in these studies. However, this protein was quantified by a total of 17 shorter, fully tryptic peptides (arginine and lysine residues marked in blue font, unique peptides underlined) from the $^{16}O/^{18}O$-labeled peptide fraction A (Supplementary Table 2). Taken together, these results illustrate the advantage of this strategy that allows separate analysis of $^{16}O/^{18}O$-labeled peptides from dually $^{16}O/^{18}O$-ICAT-labeled peptides**,** resulting in a greater number of quantified proteins.

The present labeling strategy also allows the quantitation data obtained from biotin-free $^{16}O/^{18}O$-labeled peptide fraction A and biotin-tagged $^{16}O/^{18}O$-ICAT-labeled peptide fraction B to be compared. For example, there are seven proteins (i.e. caveolin-1, flotillin-1, Lyn, Thy-1, Gamma-glutamyltranspeptidase precursor, sodium/potassium-transporting ATPase chain-1, and sarcoplasmic/endoplasmic reticulum Ca^{2+} pump) listed in Table 2 that were quantitated through peptides labeled via both techniques. In each of these cases, good correlation was observed in the abundance ratios of the proteins isolated using either Triton X-100 or Brij-96 and quantitated using either $^{16}O/^{18}O$ or dual $^{16}O/^{18}O$-ICAT labeling.

Alternatively, quantitative proteomic analysis of this type could have been carried out using conventional $^{16}O/^{18}O$ and ICAT labeling in two separate experiments[32]. Such an approach would need two identical samples that have to be separately prepared, requiring an increase in the amount of starting material and making the process much more laborious. The simultaneous use of $^{16}O/^{18}O$ and ICAT labeling on a single sample presented herein is less time-consuming and offers more efficient quantitative profiling, since collection of the avidin chromatography flow-through $^{16}O/^{18}O$-labeled fraction allows the entire sample to be utilized for proteome analysis, while collection of dually $^{16}O/^{18}O$-ICAT-labeled biotin-tagged fraction allowed analysis of smaller sample portion, resulting in the ability to quantify lower abundance proteins from the same sample in a single preparation procedure.

The effect of Triton X-100 and Brij 96 on the level of protein enrichment from DRMMs

A subset of quantified proteins that have previously been characterized as lipid raft or membrane proteins is shown in Table 2. RBL-2H3 cells express a high level of Thy-1 (GPI-anchored protein), which interacts with Lyn and forms detergent-insoluble protein complexes[21, 23, 33]. These two proteins serve as good markers for DRMM isolation since they are highly abundant within these structures in RBL-2H3 cells. Indeed, the results for Lyn and Thy-1 (Table 2) show that these proteins were quantified by multiple peptides, 55 and 45 respectively, placing them as the 22^{nd} and 36^{th} in the data set of the 738 unique proteins ranked by descending order of the total number of identified peptides per protein. The fragment ion series of both heavy and light isotopomeric peptides from Lyn indicating

their quantitation from both $^{16}O/^{18}O$-labeled and $^{16}O/^{18}O$-ICAT dually labeled peptide fractions are shown in Figs. 6B–C and 7B–C. The mass spectra of the corresponding isotopomeric peptide molecular ions, shown in Figs. 6A and 7A, together with extracted ion chromatogram of the $^{16}O/^{18}O$-labeled QLLAPGNSAGAFLIR peptide pair shown in Figure 8A, indicate a significantly higher enrichment of Lyn within Brij-96 isolated fractions, illustrated by heavy (Brij-96)/light (Triton X-100) abundance ratios of 2.12 and 2.91, obtained from both the $^{16}O/^{18}O$-labeled and the $^{16}O/^{18}O$-ICAT–labeled fractions, respectively (Table 2). This increased abundance of Lyn in DRMMs isolated using Brij-96 is confirmed by Western immunoblotting using an anti-Lyn primary antibody on low-density sucrose gradient fractions isolated by Triton X-100 and Brij-96, respectively (Fig. 8B)[23].

Based on statistical analysis applied to this data set, it was determined that the protein abundance ratios ≤0.64 and ≥1.65 were considered statistically significant for this dataset. This analysis was carried out by normalizing the number of identified peptides corresponding to their binned (e.g., 1.0–1.1, 1.1–1.2, etc.) abundance ratios calculated from their extracted ion chromatograms[34]. Using nonlinear least-squares regression analysis, it was determined that each experimentally measured heavy/light abundance ratio exhibiting two standard deviations above or below the mean demonstrates a significant difference in abundance between its isolation using Brij-96 and Triton X-100[34].

While 77 proteins showed an increased abundance when Brij-96 (i.e., abundance ratio ≥1.65) was used to isolate the DRMMs, 533 proteins showed a greater abundance in the sample isolated using Triton X-100 (i.e., abundance ratio ≤0.64). Therefore, a much greater fraction (i.e., 63.4%) of detergent-insoluble proteins was more readily isolated using Triton X-100 compared to Brij-96 (10.4%). Consequently, a total of 610 (82%) proteins showed significant changes in their concentration level in response to differential detergent isolation. These findings indicate that the solubility and corresponding abundance of proteins that were isolated have been significantly influenced by the detergent utilized during the sample extraction phase[19, 20, 23, 35, 36]. This result, however, does not suggest that Triton X-100 is definitively the better detergent for the isolation of DRMMs. For example, 71 ribosomal proteins were quantitated in this analysis indicating certain degree of crosscontamination which is unavoidably present during any of subcellular fractionation procedures and was investigated in an attempt to determine true raft components[18, 37]. The mean abundance ratio for these non-DRMMs proteins was 0.29 with a standard deviation of 0.14. This mean abundance ratio equates to ribosomal proteins being almost four times as abundant in the samples extracted using Triton X-100 compared to Brij-96. In every case (71 out of 71), the ribosomal protein was more abundant in the sample extracted using Triton X-100. These findings suggest that although Triton X-100 may provide a higher overall protein yield, its non-specificity will also result in the extraction of non-DRMMs proteins compared to Brij-96.

Conclusions

The results of this investigation validate the utility of combined sequential stable isotope labeling using chemical labeling at the protein level and enzymatic labeling at the peptide level for effective quantitative profiling of detergent-insoluble membrane proteins. This method takes advantage of both $^{16}O/^{18}O$ and ICAT labeling, coupled with buffered methanol-based solubilization and tryptic digestion of detergent-insoluble proteins. It allowed broader proteome coverage, exemplified in simultaneous quantitation of potentially lower abundance cysteine-containing peptides/proteins from biotin-tagged fraction, along with quantitation of more abundance peptides/proteins from the non-cysteine biotin-free tryptic peptide fraction, including the quantitation of non-cysteine-containing proteins.

Besides, the use of combined stable isotopic labeling on the single sample at the protein and at the peptide level significantly reduces the sample consumption. While the utility of a combined $^{16}O/^{18}O$ and ICAT labeling approach was demonstrated using DRMMs, it could be equally amenable to quantitative analysis of other cellular fractions and tissue samples. Importantly presented results confirmed the differential impact of Triton X-100 and Brij-96 on the global level of protein enrichment within respective DRMMs and can be used to optimize detergent-based isolation of a particular lipid raft protein. The results show that Triton X-100 is able to extract a greater protein yield than Brij-96; however, it also extracts greater amounts of non-DRMM components such as ribosomal proteins. The choice of detergent for DRMMs isolation must therefore be carefully considered when designing the experiment, and ultimately, the choice may depend on a specific protein target of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Synopsis

A method for quantitative profiling of detergent-resistant membrane microdomains (DRMM)s using combined ^{18}O and ICAT stable isotopic labeling is described. Comparative proteomic analysis of two differently labeled DRMM fractions, extracted from RBL-2H3 cells using Triton X-100 and Brij-96, respectively, resulted in 738 quantified proteins and confirmed the utility of combined ^{18}O/ICAT labeling as well as differential impact of these two distinct nonionic detergents on the level of protein enrichment.

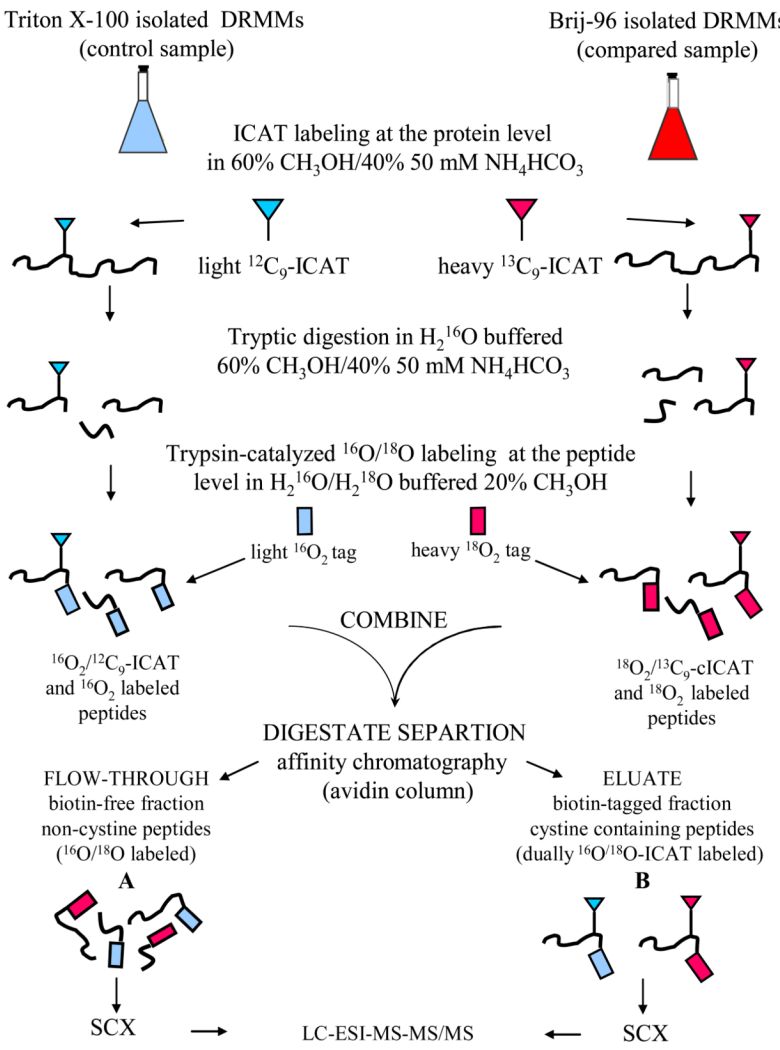

Figure 1.

The work flow of combined $^{16}O/^{18}O$ and ICAT stable isotopic labeling. Proteins from control sample (blue), and proteins from compared sample (red) were solubilized, reduced and labeled by light $^{12}C_9$-ICAT reagent (blue triangles) and heavy $^{13}C_9$-ICAT reagent (red triangles). Each sample was separately digested by direct addition of trypsin (1:20 enzyme/protein ratio) and lyophilized. The compared digestate was then reconstituted in 20% $H_2{}^{18}O$-buffered CH_3OH and trypsin-catalyzed $^{18}O_2$ labeling was carried out (red quadrangles). An analogous procedure is applied to control digestate using $H_2{}^{16}O$ buffered CH_3OH. Trypsin activity is quenched by boiling, followed by addition of 1 mM PMSF (final concentration). The control and compared digestates are combined and loaded onto a single avidin column. Dually-labeled, $^{16}O/^{18}O$-ICAT-tagged cysteine peptides are captured utilizing the biotin tag. A flow-through, $^{16}O_2/^{18}O_2$-labeled, biotin-free, peptide fraction A comprised of non-cysteinyl peptides was collected first. Subsequently, dually labeled peptides (i.e., $^{18}O_2{}^{13}C_9$-ICAT /$^{16}O_2{}^{12}C_9$-ICAT) are eluted and collected as biotin-tagged peptide fraction B. Upon collection biotin tags are cleaved according to the manufacturer's instructions. Both digestates, $^{16}O/^{18}O$-labeled and dually $^{16}O/^{18}O$-ICAT labeled were separately lyophilized and fractionated using SCX chromatography followed by reversed phase μLC-ESI-MS/MS analysis of each fraction.

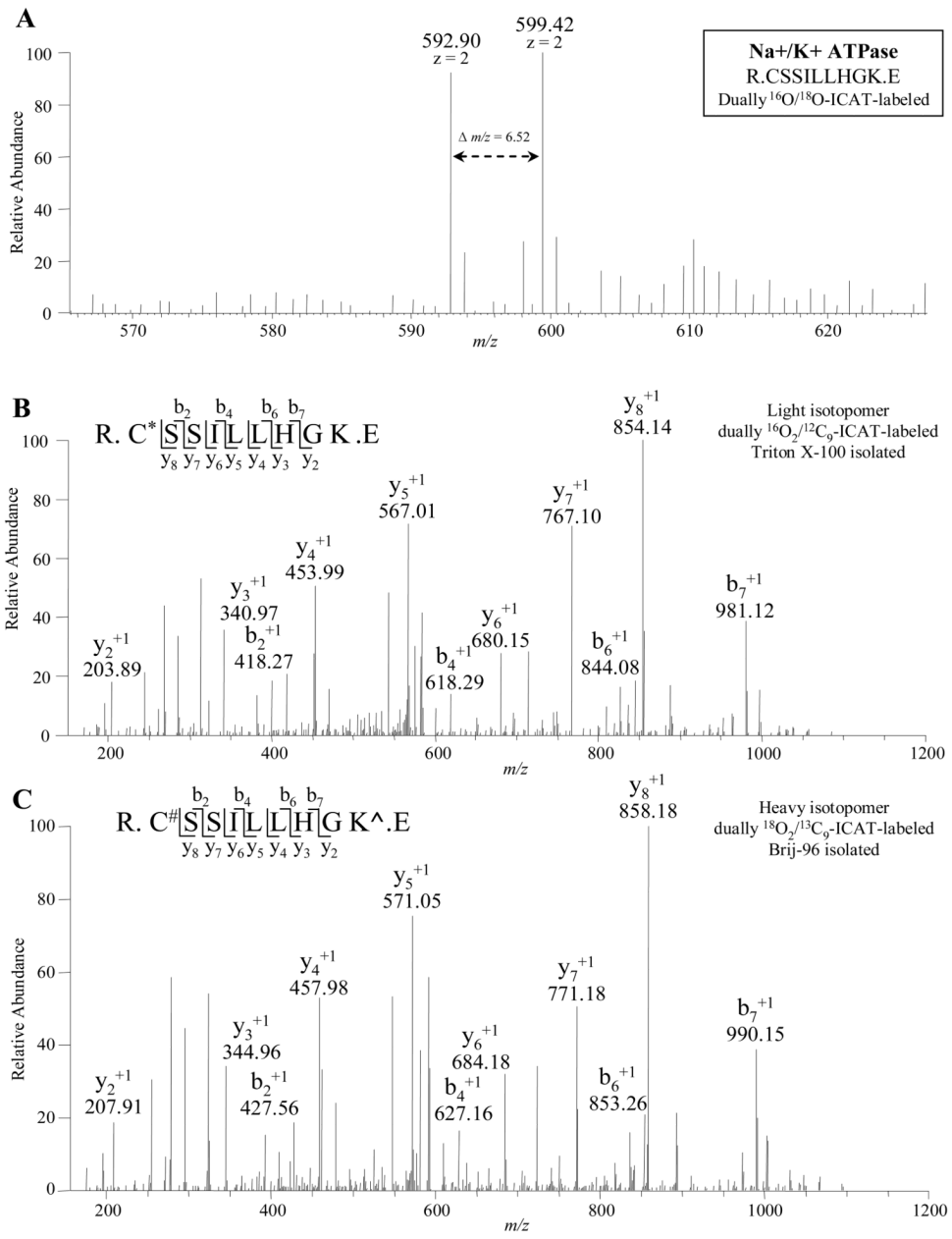

Figure 2.

(A) Zoomed portion of mass spectrum showing the $^{16}O_2{}^{12}C_9$-ICAT/ $^{18}O_2{}^{13}C_9$-ICAT dually labeled, $[M+2H]^{2+}$ molecular-ion pair (acquired using quadrupole ion trap) eghibiting typical mass-to-charge (*m/z*) ratio difference of 6.52 Da for the labeling technique described herein. The *m/z* ratio difference between chemically identical but by mass different peptides depends on peptide charge state and the mass difference (shift) between light and heavy isotope tags. The calculated mass difference of 13.038 Da between singly charged, $^{16}O^{18}O$-ICAT dually labeled isotopomeric peptides is the sum of the 4.008 Da difference between $^{18}O_2$ and $^{16}O_2$ modified c-terminus and 9.030 Da differences between $^{13}C_9$-ICAT and $^{12}C_9$-ICAT-modified single cysteine residue. **(B)** Tandem mass spectrum of the precursor ion m/z = 592.90 $[M+2H]^{2+}$ identifying the sequence of the light $^{16}O_2/^{12}C_9$-ICAT-labeled isotopomer C*SSILLHGK. Y-fragment ion series indicates unmodified c-

terminus, while b-fragment ions indicate $^{12}C_9$-ICAT-modified cysteinyl residue marked as C* of tryptic peptide from sodium/potassium-transporting ATPase. **(B)** MS/MS spectrum of the m/z = 599.42 $[M+2H]^{2+}$ precursor ion, shows identification of heavy $^{18}O_2/^{13}C_9$-ICAT-labeled isotopomer $C^{\#}$SSILLHGK^ where y-fragment ions reveal $^{18}O_2$ modified c-terminus marked with ^. Correspondingly, b-fragment ions indicate modification of cysteinyl residue marked as $C^{\#}$ by the heavy $^{13}C_9$-ICAT tag. Observed shift of ~4 Da between analogous $[M+1H]^{1+}$ y-fragments series of light (A) and heavy (B) isotopomer confirm stability of c-terminal modification, while m/z differences of ~9 Da between analogous $[M+1H]^{1+}$ b-fragment series verify stability of modified cysteine residue.

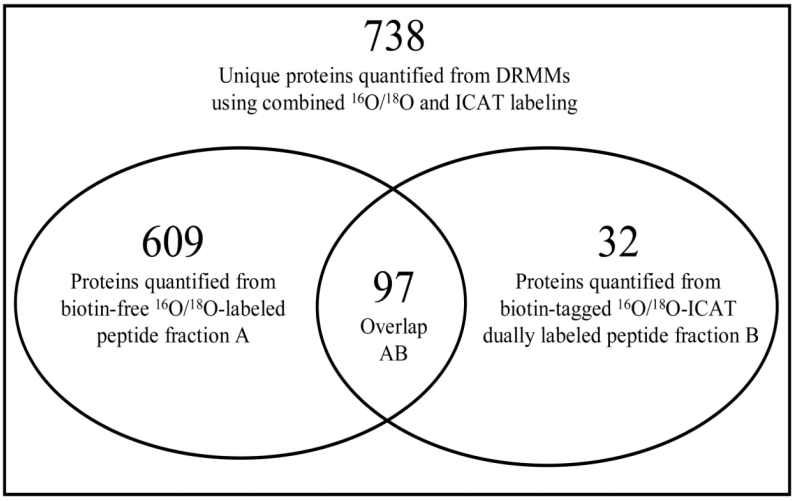

Figure 3.

Venn diagram illustrating the relationship between the total number of quantified proteins from detergent-resistant membrane domains of RBL-2H3 cells using combined $^{16}O^{18}O$ and cICAT labeling and the numbers of protein quantified from the $^{16}O/^{18}O$-labeled peptide fraction A and the dually $^{16}O/^{18}O$-ICAT-labeled peptide fraction B, respectively.

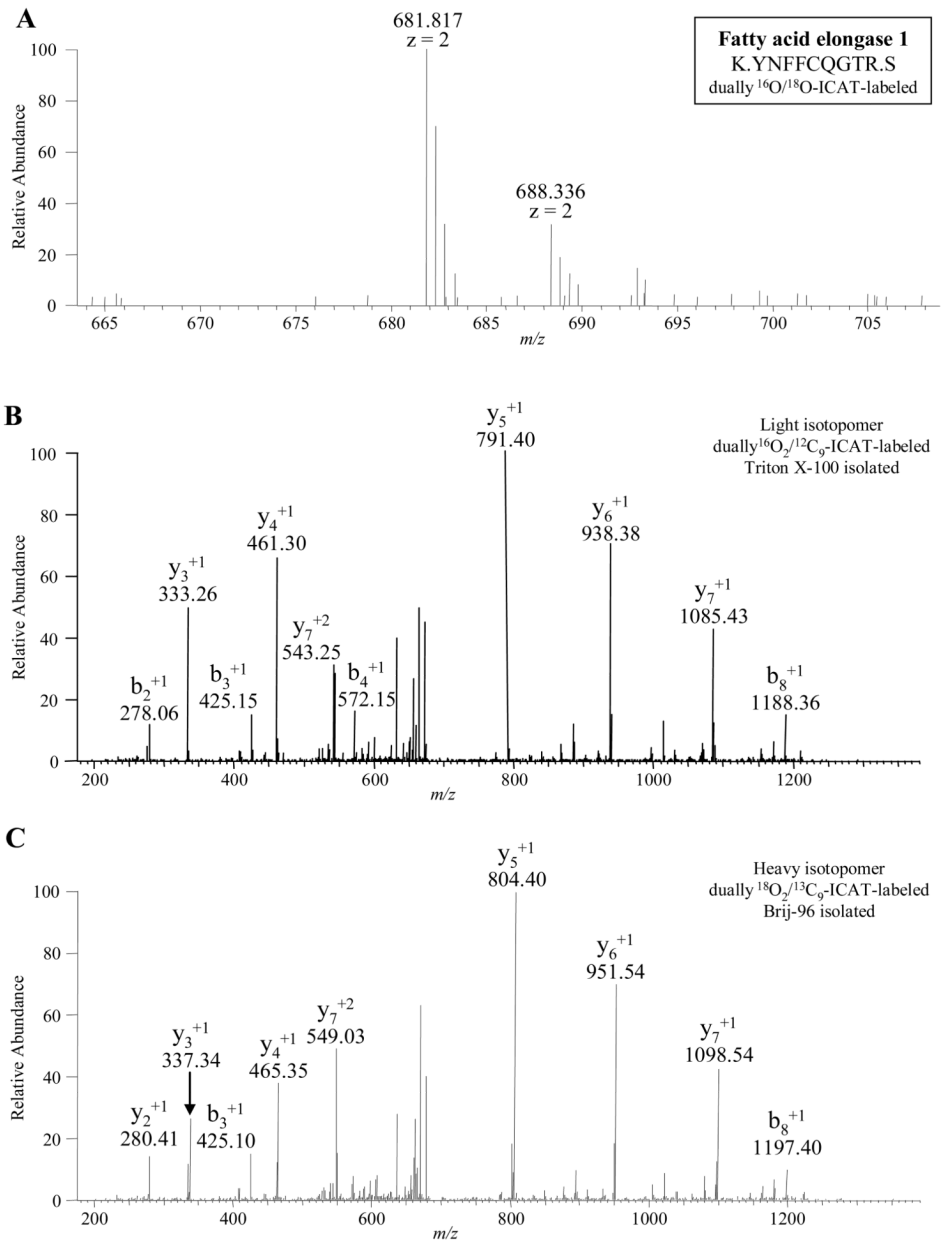

Figure 4.

Selected mass spectra acquired by hybrid LIT-FTICR mass spectrometer of dually $^{16}O/^{18}O$-ICAT-labeled molecular ion pair, representing light and heavy isotopomeric peptide (YNFFCQGTR) from fatty acid elongase. **(A)** Zoomed portion of the FTICR mass spectrum showing a pair of doubly charged molecular ions (light: m/z = 681.817 $[M+2H]^{2+}$, heavy: m/z = 688.336 $[M+2H]^{2+}$, Δ *m/z* = 6.519 Da), indicating lower abundance of heavy labeled isotopomeric peptide. **(B)** Tandem LIT mass spectrum of precursor ion m/z = 681.817 $[M+2H]^{2+}$ shown in A illustrates identification of light $^{16}O_2/^{12}C_9$-ICAT-labeled isotopomeric peptide. **(C)** Tandem LIT mass spectrum of precursor ion m/z = 688.336 $[M+2H]^{2+}$, shown in A illustrates identification of heavy $^{18}O_2/^{13}C_9$-ICAT-labeled isotopomeric peptide.

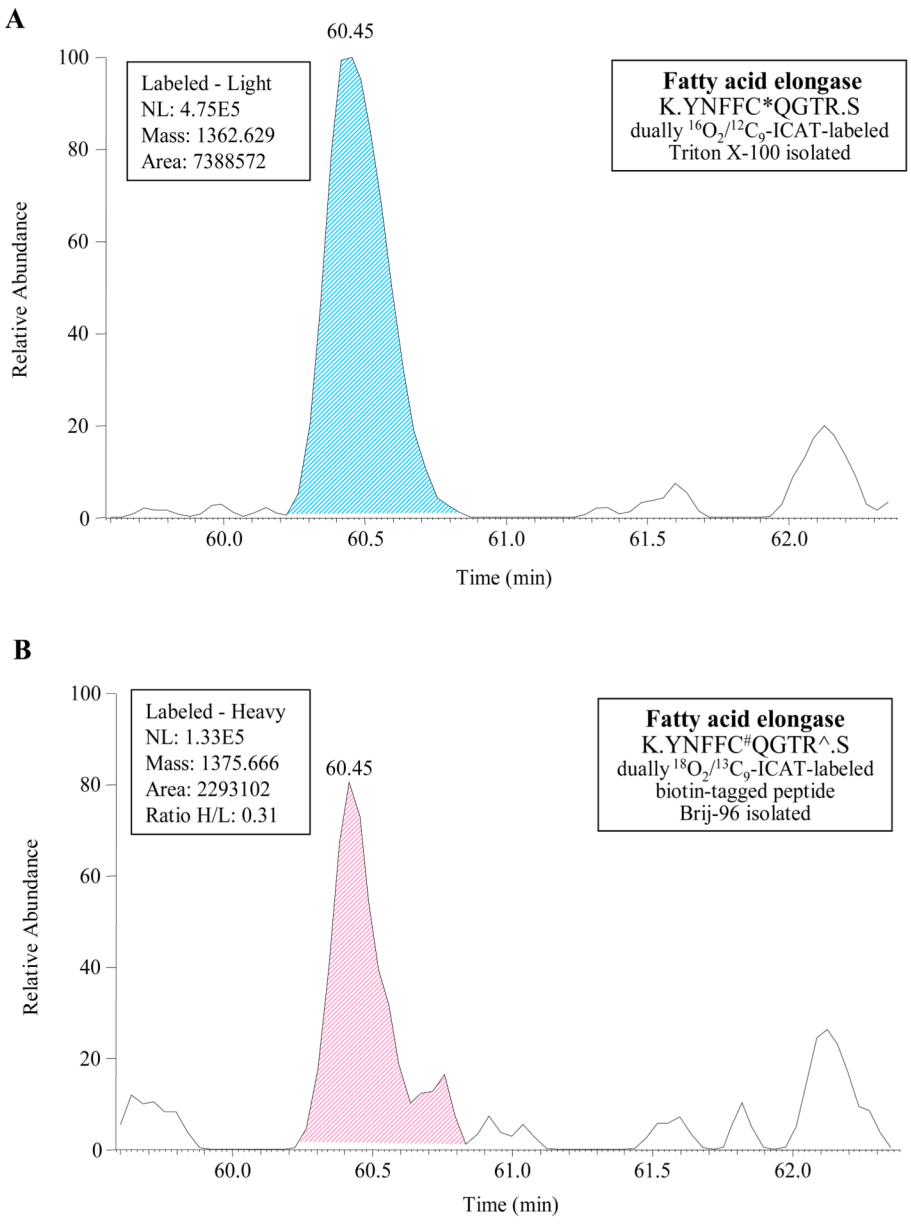

Figure 5.

Relative quantitation of dually $^{16}O/^{18}O$-ICAT-labeled isotopomeric peptides (shown in Figure 4) from Triton X-100 and Brij-96 isolated samples indicating higher enrichment (Ratio H/L; 0.31) of fatty acid elongase from Triton X-100 isolated sample. **(A)** Reconstructed ion chromatograms of the precursor ion *m/z* = 681.817 $[M+2H]^{2+}$ (shown in Fig. 4A), representing the light $^{16}O_2/^{12}C_9$-ICAT-labeled isotopomeric peptide (calculated mass = 1362.6289 Da $[M+H]^{1+}$). **(B)** Reconstructed ion chromatograms of the precursor ion *m/z* = 688.336 $[M+2H]^{2+}$ (shown in Fig. 4A) representing the heavy $^{18}O_2/^{13}C_9$-ICAT-labeled isotopomeric peptide (calculated mass = 1375.6669 Da $[M+H]^{1+}$).

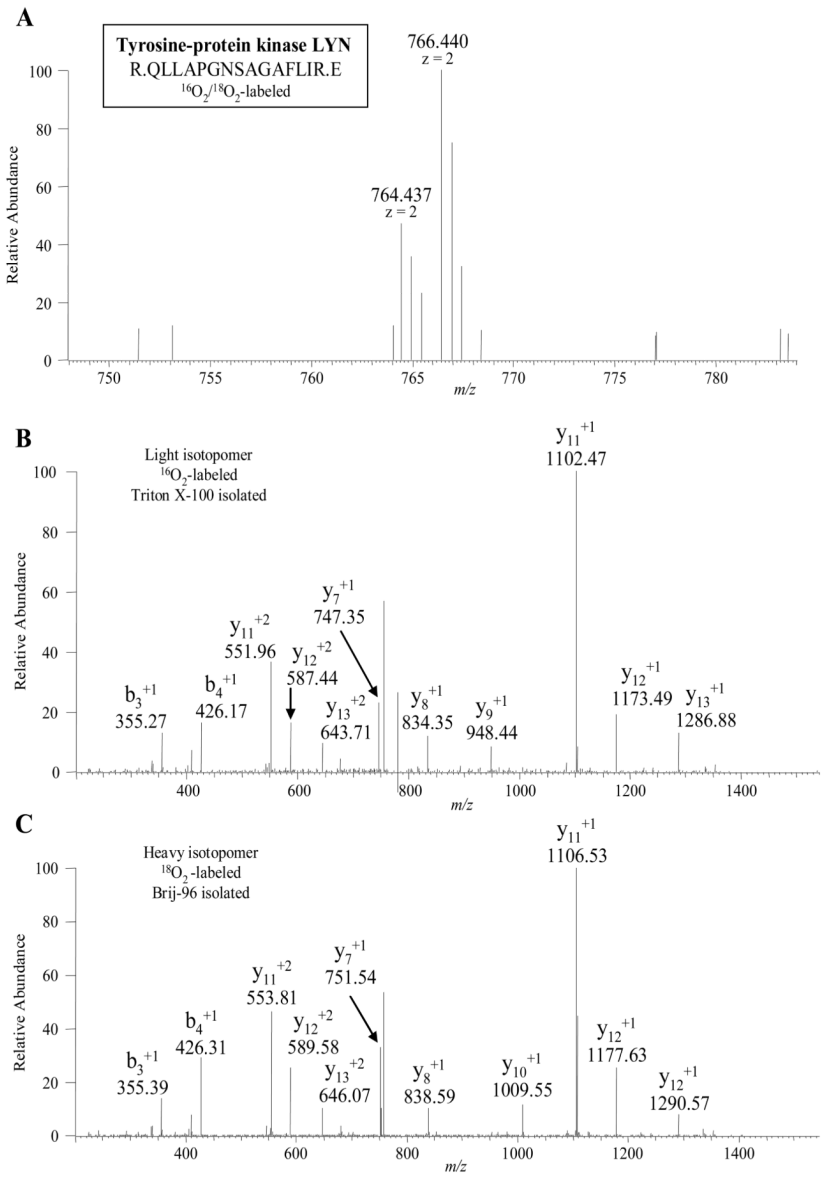

Figure 6.

Selected mass spectra of $^{16}O/^{18}O$-labeled molecular ion pair (biotin-free peptide fraction), representing light and heavy isotopomeric peptide (QLLAPGNSAGAFLIR) from tyrosine protein kinase LYN. **(A)** Zoomed portion of the FTICR mass spectrum showing a pair of doubly charged molecular ions (light: m/z = 764.437 $[M+2H]^{2+}$, heavy: m/z = 766.440 $[M+2H]^{2+}$, Δ *m/z* = 2.003 Da), indicating higher abundance of heavy labeled isotopomeric peptide. **(B)** Tandem LIT mass spectrum of precursor ion m/z = 764.437 $[M+2H]^{2+}$ shown in A illustrates identification of light $^{16}O_2$ labeled isotopomeric peptide.**(C)** Tandem LIT mass spectrum of precursor ion m/z = 766.440 $[M+2H]^{2+}$ shown in A illustrates identification of heavy $^{18}O_2$-labeled isotopomeric peptide as indicated by the difference of ~4 Da between corresponding y $[M+H]^{1+}$fragment ions of heavy and light isotopomeric peptides shown in C and B, respectively.

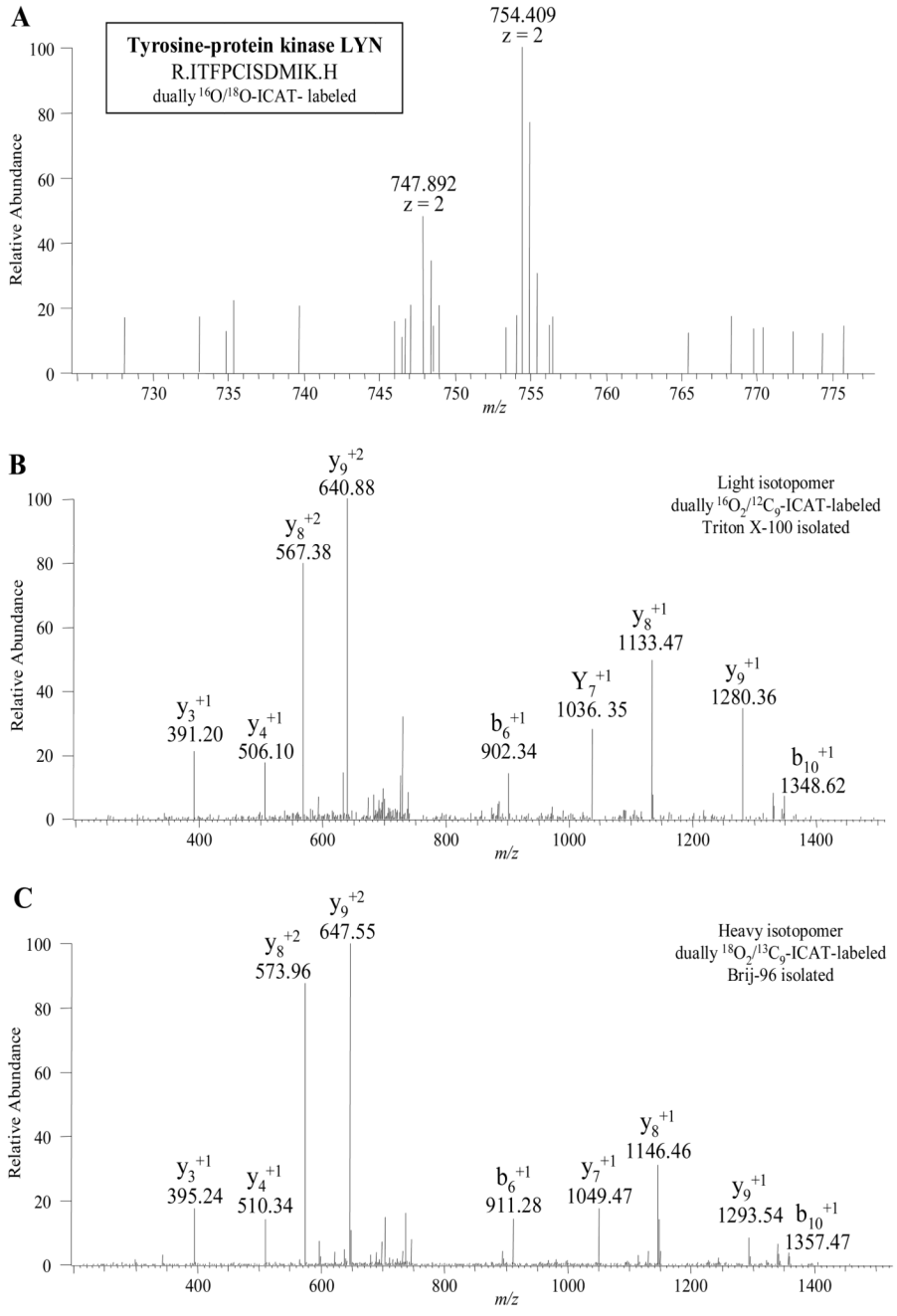

Figure 7.

Selected mass spectra of dually $^{16}O/^{18}O$-ICAT-labeled molecular ion pair (biotin-tagged peptide fraction A), representing light and heavy isotopomeric peptide (ITFPCISDMIK) from tyrosine protein kinase LYN. **(A)** Full-range FTICR mass spectrum showing a pair of doubly charged molecular ions (light: m/z = 747.890 $[M+2H]^{2+}$, heavy: m/z = 754.409 $[M+2H]^{2+}$, Δ *m/z* = 6.519 Da), indicating higher abundance of heavy labeled isotopomeric peptide. **(B)** Tandem LIT mass spectrum of precursor ion m/z = 747.890 $[M+2H]^{2+}$ shown in A illustrates identification of light $^{16}O_2/^{12}C_9$-ICAT-labeled isotopomeric peptide.**(C)** Tandem LIT mass spectrum of precursor ion m/z = 754.409 $[M+2H]^{2+}$, shown in A illustrates identification of heavy $^{18}O_2/^{13}C_9$-ICAT-labeled isotopomeric peptide.

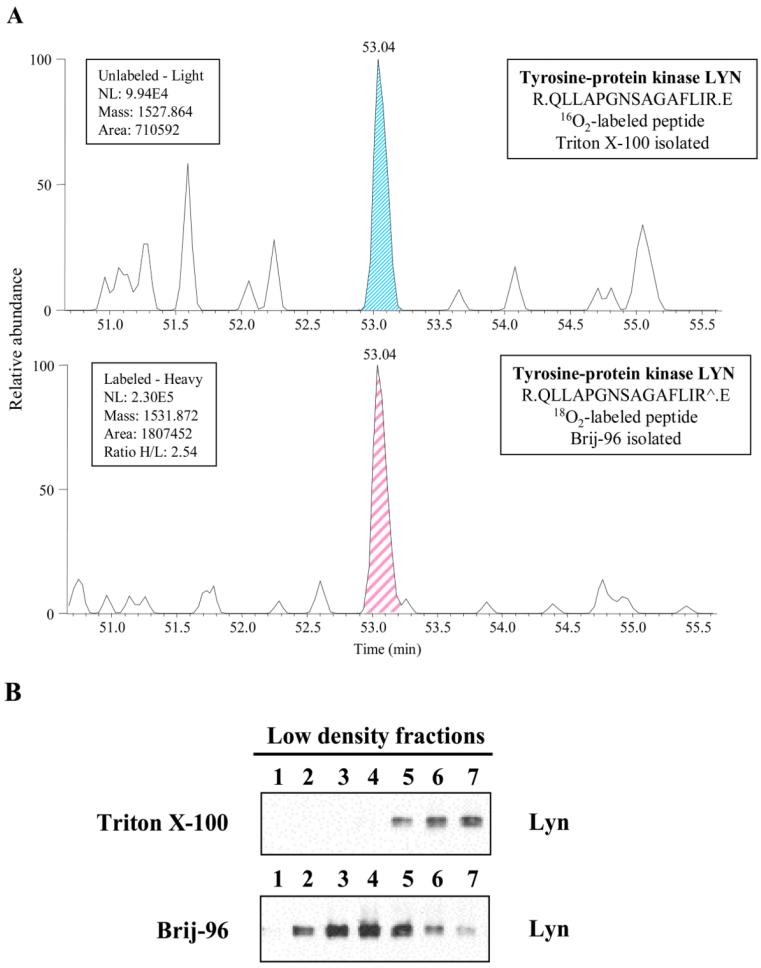

Figure 8.

Relative quantitation of tyrosine protein kinase Lyn using proteomic, solution-based stable isotope labeling and the Western immunoblot from differently isolated (Triton X-100 and Brij-96) DRMMs, confirming higher enrichment of tyrosine protein kinase Lyn in Brij-96 isolated fraction by both techniques. **(A)** Reconstructed ion chromatograms of light (*m/z* = 764.437 $[M+2H]^{2+}$) and heavy (*m/z* = 766.440 $[M+2H]^{2+}$) precursor ion shown in Figure 6A, representing the light $^{16}O_2$ labeled (calculated mass = 1527.8642 Da $[M+H]^{1+}$) and the heavy (calculated mass = 1531.8727 $[M+H]^{1+}$) isotopomer of the QLLAPGNSAGAFLIR peptide. **(B)** Tyrosine protein kinase Lyn levels measured by Western immunoblot from the low-density sucrose gradient fractions isolated using Triton X-100 and Brij-96 respectively, indicating higher yield (enrichment) of tyrosine protein kinase Lyn in Brij-96 isolated fractions.

Table 1

Quantitation of differentially $^{16}O/^{18}O$-ICAT dually labeled, fully tryptic peptides (i.e., light $^{16}O_2{}^{12}C_9$-ICAT-labeled isotopomer and heavy $^{18}O_2{}^{13}C_9$-ICAT-labeled isotopomer) from sodium/potassium-transporting ATPase chain-1, identified from identically extracted DRMMs using Triton X-100 and mixed using 1:1 protein ratio.

PEPTIDE *A*	OR*B*	AR*C*	SD*D*	[M+1H]$^{1+}$*E*	CHG*F*	X_{CORR}*G*
R.C^* SSILLHGK.E	1.08			1184.6486	2	2.9054
R.$C^\#$ SSILLHGK^.E	1.08			1197.6786	2	2.6824
K.AC^* VVHGSDLK.D	1.19			1255.6493	2	3.1774
K.A$C^\#$ VVHGSDLK^.D	1.19			1268.6793	2	3.3512
		1.13	0.07			

A Amino acid sequence of identified peptide.

C^* indicates cysteinyl residue labeled with light $^{12}C_9$-ICAT reagent.

$C^\#$ indicates cysteinyl residue labeled with heavy $^{13}C_9$-ICAT reagent.

^ indicates ^{18}O-modified C-terminus.

B Observed ratio (i.e., Triton X-100 extracted experiment 1 divided by Triton X-100 experiment 2 for the individual peptides)

C Average protein abundance ratio;

D Standard deviation;

E Calculated mass [M+1H]$^{1+}$ of identified peptide;

F Charge state of detected precursor ion;

G SEQUEST cross-correlation score.

Table 2

Sub-set of quantified proteins from RBL-2H3 cells DRMMs differentially extracted employing Triton X-100 and Brij-96 respectively, tagged using combined $^{16}O/^{18}O$ and ICAT stable isotope labeling followed by avidin chromatography separation of biotin-free $^{16}O/^{18}O$-labeled peptide fraction A from biotin-tagged dually $^{16}O/^{18}O$-ICAT-labeled peptide fraction B and consecutive fractionation by SCX chromatography, followed by nanoflow RPLC-ESI-MS/MS analysis.

P A N[A]	FID[B]	PROTEIN NAME AND PEPTIDE SEQUENCE[C]	AR[D]	SD[E]	T/U[F]	Ch/Xc[G]	TM[H]
P23347	**A**	**Anion exchange protein 2**			**9/4**		**11**
	A	K.LSVPSGFSVTAPDKR^.G	1.07	0.06	9/4	2/2.51	
P41350	**AB**	**Caveolin-1**			**14/4**		**2**
	A	K.HLNDDVVK#.I	0.21	0.06	13/3	2/2.99	
	B	K.SFLIEIQ[C*] ISR.V	0.36		1/1	2/3.19	
P61805	**A**	**Defender against cell death 1 (dad-1)**			**17/4**		**3**
	A	K.ADFQGISPER^.A	0.76	0.17	17/4	2/2.51	
P11167	**A**	**Facilitated glucose transporter type 1**			**15/3**		**12**
	A	R.TFDEIASGFR.Q	0.04	0.01	15/3	2/4.37	
Q920L7	**B**	**Fatty acid elongase 1**			**3/2**		**7**
	B	K.YNFF[C#] QGTR^.S	0.31	0.05	3/2	2/2.45	
Q9Z1E1	**AB**	**Flotillin-1**			**34/14**		**0**
	A	R.LTGVSISQVNHNKPLR^.T	0.24	0.09	32/12	2/2.92	
	B	R.VFVLP[C*] IQQIQR.I	0.15	0.04	2/2	2/3.48	
P07314	**AB**	**Gamma-glutamyltranspeptidase 1 precursor**			**10/5**		**1**
	A	R.KAEVINAR^.E	2.26	0.64	8/4	2/2.69	
	B	K.TPAL[C#] EVF[C#] R^.Q	2.25	0.00	2/1	2/2.33	
Q924B5	**AB**	**Glycoprotein TEC-21**			**16/4**		**0**
	A	K.TAILASK^.S	0.26	0.12	11/2	2/2.23	
	B	K.[C*] NPGEF[C*] QETVLLIK.A	0.16	0.09	5/2	2/3.81	
O35165	**A**	**Golgi SNARE protein (Membrin)**			**7/3**		**1**
	A	K.YDVQHLQTALR^.N	0.76	0.37	7/3	2/3.71	

P A N[A]	FID[B]	PROTEIN NAME AND PEPTIDE SEQUENCE[C]	AR[D]	SD[E]	T/U[F]	Ch/Xc[G]	TM[H]
P06762	**A**	**Heme oxygenase 1 (HSP32)**			**14/5**		**1**
	A	R.LHEVGGTHPELLVAHAYTR^.Y	0.48	0.19	14/5	2/4.95	
Q64273	**B**	**Inward rectifier K^+ channel 2**			**2/1**		**2**
	B	K.TYEVPNTPL^{C*} SAR.D	0.10	0.00	2/1	2/3.48	
P53987	**A**	**Monocarboxylate transporter 1 (MCT 1)**			**51/8**		**11**
	A	R.DGKEDETSTDVDEKPK.K	0.08	0.04	51/8	2/5.02	
P11507	**AB**	**Sarcoplasmic/endoplasmic reticulum Ca^{2+} pump**			**5/5**		**8**
	A	R.WGSNELPAEEGK.T	0.20	0.17	4/4	2/3.76	
	B	R.ANA^{C*} NSVIK.Q	0.13		1/1	2/2.27	
P06685	**AB**	**Sodium/potassium-transporting ATPase chain-1**			**57/18**		**10**
	A	K.VDNSSLTGESEPQTR^.S	1.28	0.29	52/16	2/5.11	
	B	K.LIIVEG$^{C\#}$ QR^.Q	0.94	0.32	5/2	2/2.51	
P01830	**AB**	**Thy-1 membrane glycoprotein precursor**			**45/10**		**0**
	A	K.HVLSGTLGVPEHTYR^.S	0.63	0.21	44/9	2/5.51	
	B	R.VISLTA$^{C\#}$ LVNQNLR^.L	0.59		1/1	2/3.46	
Q07014	**AB**	**Tyrosine-protein kinase LYN**			**55/17**		**0**
	A	R.QLLAPGNSAGAFLIR^.E	2.12	0.29	53/15	2/3.97	
	B	R.ITFP$^{C\#}$ ISDMIK^.H	2.91	0.71	2/2	2/2.44	

[A] Swiss-Prot primary accession number;

[B] Fraction in which protein (bold font) or peptide (regular font) was identified;

[C] Bold font, protein description. Regular font, sequences of selected quantified peptides.

[C*] indicates cysteinyl residue labeled with light $^{12}C_9$-ICAT reagent.

[C#] indicates cysteinyl residue labeled with heavy $^{13}C_9$-ICAT reagent.

^ indicates ^{18}O-modified c-terminus;

[D] Average abundance ratio is based on calculation of heavy (Brij-96 isolated) to light (Triton X-100 isolated) ratio;

[E] Standard deviation of AR;

[F] Bold font indicates a total number of peptides quantified vs. a total number of unique peptides quantified for a particular protein. Regular font: total peptides/unique peptides quantified from either the biotin-free fraction A or biotin-tagged fraction B;

[G] Precursor ion charge state and SEQUEST cross-correlation score;

[H] Number of mapped transmembrane domains.

Table 3

Sub-set of cysteine-containing membrane proteins quantified solely from biotin-tagged $^{16}O/^{18}O$-ICAT dually labeled peptide fraction B of differently isolated DRMMs using Triton X-100 and Brij- 96.

PAN[A]	Protein description	AR[B]
Q6JWR2	Amino acid transporter	3.31
Q99PE7	ATP-binding cassette, sub-family G, member 5 (Sterolin-1)	1.05
Q62745	CD81 antigen (26 kDa cell surface protein TAPA-1)	0.13
Q8VII6	Choline transporter-like protein 1, splice variant a	0.53
Q6P4Z8	Dolichyl-phosphate GlcNAc-1-P transferase	0.15
Q920L7	Fatty acid elongase 1	0.31
P54313	Guanine nucleotide-binding protein	1.06
P30033	Guanine nucleotide-binding protein G(o), alpha subunit 2	1.53
Q5U2Z8	Hypothetical protein	0.57
Q64273	Inward rectifier potassium channel 2	0.10
P18163	Long-chain-fatty-acid--CoA ligase	0.10
Q63002	Mannose 6-phosphate/insulin-like growth factor II receptor	0.48
Q9R0T3	Protein kinase inhibitor p58	0.39
P16391	RT1 class I histocompatibility antigen	1.87
P10960	Sulfated glycoprotein 1 precursor (SGP-1)	0.07

[A] Swiss-Prot primary accession number,

[B] Abundance ratio.

Table 4

Sub-set of membrane proteins which possess no cysteinyl residues and were quantified exclusively by $^{16}O/^{18}O$ labeling from biotin-free peptide fraction A of differently isolated DRMMs using Triton X-100 and Brij-96.

PAN[A]	Protein description	AR[B]
Q6AXS4	Atp6ap2 protein (Renin/prorenin receptor)	0.19
P70629	B-cell receptor-associated protein	2.38
P00173	Cytochrome b5	0.08
P35171	Cytochrome c oxidase subunit VIIa-L	3.21
Q63190	Emerin	0.47
P06762	Heme oxygenase 1(HO-1)	0.48
Q5U2V8	MGC94540 protein	2.91
Q9WTR7	Microsomal signal peptidase 21	1.99
Q6PDU7	Similar to CG6105-PA	0.37
Q08849	Syntaxin-3	1.55
Q08013	Translocon-associated protein, gamma subunit	0.47
P63081	Vacuolar ATP synthase 16 kDa proteolipid subunit	0.26

[A] Swiss-Prot primary accession number,

[B] Abundance ratio.