Article

SWATH-Based Comprehensive Determination of the Localization of Apical and Basolateral Membrane Proteins Using Mouse Liver as a Model Tissue

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Abstract:
The purpose of this study was to develop a method to comprehensively determine the localization of apical and basolateral membrane proteins, using a combination of apical/basolateral membrane separation and accurate SWATH (Sequential Window Acquisition of all Theoretical fragment ion spectra) proteomics. The SWATH analysis of basolateral and apical plasma membrane fractions in mouse liver quantified the protein expression of 1373 proteins. The basolateral/apical ratios of the protein expression levels were compared with the reported immunohistochemical localization for 41 model proteins (23 basolateral, 11 apical and 7 both membrane-localized proteins). Three groups were perfectly distinguished. Border lines to distinguish the apical-, both- and basolateral localizations were determined to be 0.766 and 1.42 based on probability density. The method that was established was then applied to the comprehensive determination of the proteins in mouse liver. The findings indicated that 154 and 125 proteins were localized in the apical and basolateral membranes, respectively. The levels of receptors, CD antigens and integrins, enzymes and Ras-related molecules were much higher in apical membranes than in basolateral membranes. In contrast, the levels of adhesion molecules, scaffold proteins and transporters in basolateral membranes were much higher than in apical membranes.

Keywords: basolateral membrane; apical membrane; membrane localization; comprehensive quantitative proteomics; SWATH

1. Introduction

Tissues harbor two types of cell membranes, namely, apical and basolateral membranes. The membrane facing basal lamina and neighboring cells is referred to as the “basolateral membrane”. The membrane on the opposite side of the basolateral membrane in polarized cells is referred to as the “apical membrane”. Elucidating which proteins are expressed on which membranes is important in many areas of biology. For example, transporters typically transport substances in a fixed direction, and the direction of transport can be 180 degrees different depending on which membrane they are expressed on. Therefore, to understand the physiological and pharmacological roles of an organ or a cell, it is essential to understand the localization of the transporter. In terms of drug delivery, liposomes and other drug products that are administered via the bloodstream are taken up from the cell membrane on the blood side into the cells of each organ. For active targeting, membrane proteins that are localized on the blood side of the cell membrane need to be targeted. In contrast, to avoid side effects, it is desirable to target molecules that are not localized on the blood side of the cell membrane. In order to achieve this, it is important to have available a comprehensive list of membrane proteins that are localized on apical and basolateral cell membranes in each organ. This task is best suited to comprehensive profiling approaches such as proteomics, rather than laborious immunohistochemical analysis.
Since the liver is the organ where drug delivery system (DDS) products such as liposomes tend to accumulate, it is important that the products are not easily taken up by liver cells in order to avoid toxicity. The ASGPR receptor is localized on the blood side of the cell membrane of liver parenchyma cells. The uptake of DDSs that target this receptor on liver cells are dramatically increased [1]. In contrast, the surface binding of folic acid has been reported to reduce the uptake of DDS products into the liver [2]. We have only limited knowledge regarding the localization of folate receptors and transporters in the liver, but our current knowledge suggests that they may be localized to apical membranes (not the blood side). Thus, a comprehensive determination of the localization of many proteins on basolateral and apical membranes would accelerate the development of drugs that have minimal or no liver side effects or are targeted to the liver.

The SWATH method is one of the more recent comprehensive quantitative proteomics methods that have been developed, and its excellent quantitative accuracy as compared with previous comprehensive proteomics is a significant advantage [3]. Multiple specific peptides derived from a single protein can be quantified, and the change in the level of expression of a target protein can be accurately quantified based on the average of these peptides. Membrane proteins contain hydrophobic regions, such as transmembrane sites, which cause their incomplete solubilization and resistance to trypsin digestion. However, we have previously reported on an improvement in the accuracy of the SWATH method by completely solubilizing such proteins with guanidine hydrochloride, thus improving the efficiency of the tryptic digestion of membrane proteins [4], and by applying in silico peptide selection criteria [5], such as excluding transmembrane sites and sequences that are known to be poorly digested by trypsin from the numerous peptides that are measured [6,7]. Furthermore, it has been known for a long time that apical and basolateral membranes can be fractionated from tissue samples based on the difference in the density of the cell membrane [8]. We hypothesized that, if the SWATH analysis could be applied to fractions of apical and basolateral membranes fractionated from each organ, it would be possible to comprehensively and accurately determine the localization of many membrane proteins in a variety of organs.

The purpose of this study was to demonstrate that by employing a combination of plasma membrane fractionation and the SWATH method, it would be possible to comprehensively and accurately determine the apical/basolateral localization of membrane proteins, using mouse liver as a model organ. In this study, a list of proteins localized on the apical and basolateral membranes in mouse liver was generated by this comprehensive localization analysis.

2. Materials and Methods

2.1. Animals

Male ddY mice were purchased from Charles River (Yokohama, Japan). The mice were maintained on a 12 h light/dark cycle in a temperature-controlled environment with free access to food and water. ddY mice were used at 10 weeks of age. The animal experiments were conducted based on ARRIVE guidelines, and the protocol was approved by the Institutional Animal Care and Use Committee at Tohoku University.

2.2. Separation of Basolateral (Sinusoidal) and Apical (Canalicular) Plasma Membranes of Mouse Liver by Density-Gradient Ultracentrifugation

The separation of basolateral and apical plasma membranes was performed as previously described with minor modifications [4]. The mouse liver was excised after PBS perfusion from the heart under anesthesia and were minced well with scissors on ice and homogenized by 10 up-and-down rotated strokes (1000 rpm) of a Potter-Elvehjem glass homogenizer in 40 mL of hypotonic buffer (10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (1% (v/v), Sigma Chemical Co., St. Louis, MO, USA)) per g wet tissue on ice. After incubation for 30 min on ice, 20 up-and-down strokes with rotation (1000 rpm, 4 °C) were applied.
The resulting homogenate was subjected to nitrogen cavitation at 800 psi for 15 min at 4 °C twice. The resulting homogenate was centrifuged at 8000 × g for 10 min at 4 °C and the resulting supernatant was centrifuged at 100,000 × g for 60 min at 4 °C. The pellet was suspended in suspension buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4), layered on top of a 38% (w/v) sucrose density gradient solution and centrifuged at 100,000 × g for 40 min at 4 °C. The turbid layer was transferred, recovered in suspension buffer and centrifuged at 100,000 × g for 40 min at 4 °C. The resultant pellet (used as the plasma membrane fraction) was suspended in 4 mL of suspension buffer and homogenized using a 10 mL glass homogenizer (50 up-and-down strokes with rotation at 4 °C). The homogenate was layered on top of a 31%/34%/38% (w/v) sucrose density gradient solution, and centrifuged at 195,700 × g for 3 h at 4 °C. The two turbid layers at the interfaces (the first layer, top/31%, apical plasma membrane fraction; the third layer, 34%/38%, basolateral plasma membrane fraction) were recovered, and each was diluted in suspension buffer and centrifuged at 100,000 × g for 40 min at 4 °C. The resultant pellets were suspended in suspension buffer to give the individual membrane fractions. The Lowry method with the DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure protein concentrations. The membrane fractions were stored at −80 °C.

2.3. Sample Preparation for SWATH-Based Quantitative Proteomics

Protein digestion of basolateral and apical membrane fractions prepared from four mouse livers was performed using 50 µg of protein per tube as described previously [9]. The tryptic digests were cleaned up with a self-packed SDB-XD 200 µL tip (3M, Maplewood, MN, USA), as previously described [10].

2.4. LC-MS/MS Measurement for SWATH-Based Quantitative Proteomics

The cleaned peptide samples (1 µg peptide) were injected into an NanoLC 425 system (Eksigent Technologies, Dublin, CA, USA) coupled with an electrospray-ionization Triple TOF 5600 mass spectrometer (SCIEX, Framingham, MA, USA), which was set up for a single direct injection and analyzed by SWATH-MS acquisition, as previously described [3] with minor modifications. The peptides were directly loaded onto a self-packed 20 cm long C18 analytical column, prepared by packing ProntoSIL 200-3-C18 AQ beads (Catalogue number 0001H184PS030, 3 µm, 120Å, BISCHOFF Chromatography, Germany) in a PicoFrit tip (ID 75 µm, Catalogue number PF360-75-10-N5, New Objective, Littleton, MA, USA). After sample loading, the peptides were separated and eluted with a linear gradient; 98% A: 2% B to 65% A: 35% B (0–120 min), increase to 0% A: 100% B (120–121 min), maintained at 0% A: 100% B (121–125 min), reduced to 98% A: 2% B (125–126 min) and then maintained at 98% A: 2% B (126–155 min). The composition of Mobile phase A was 0.1% formic acid in water, and that for mobile phase B was 0.1% formic acid in acetonitrile. The flow rate was 300 nL/min. The eluted peptides were positively ionized and measured in the SWATH mode. The measurement parameters were as follows: SWATH window, 64 variable windows from 400 m/z to 1200 m/z; product ion scan range, 50–2000 m/z; declustering potential, 100; rolling collision energy value, 0.0625 × [m/z of each SWATH window] − 3.5; collision energy spread, 15; accumulation time, 0.05 s for each SWATH window.

2.5. Data Analysis for SWATH-Based Quantitative Proteomics

Spectral alignment and data extraction from the SWATH chromatogram (uploaded to the Peptide Atlas website with Identifier PASS01726) were performed with the SWATH Processing Micro App in Peakview (SCIEX) using in-house spectral libraries (uploaded to the Peptide Atlas website with Identifier PASS01726), as previously described [7]. The parameters for peak data extraction by Peakview were as follows: number of peptides per protein, 999; number of transitions per peptide, 6; peptide confidence threshold, 99%; false discovery rate (FDR) threshold, 1.0%; XIC extraction window, ±4.0 min; XIC width (ppm), 50. According to a previously described procedure [6], unreliable peaks and peptides were removed based on the criteria of data selection and amino acid sequence-based
peptide selection, and the peak areas at the peptide level were calculated as an average of those in the transition level after normalizing the differences in signal intensity between the different transitions. The peptide selection criteria are described in Table S1. The details were reported in our previous study [6]. The peak areas of individual proteins were calculated as an average of those at the peptide level, and were used to calculate the basolateral/apical (B/A) ratio. The mean and SEM of four mice were calculated. Subcellular location information for all the proteins quantified was obtained from the uniprot database.

3. Results

3.1. Validation of the SWATH-Based Comprehensive Determinations of Basolateral and Apical Plasma Membrane Localizations

SWATH analysis of basolateral and apical plasma membrane fractions in mouse liver showed that 1373 proteins were quantified (Table S2). Basolateral/Apical (B/A) ratios and subcellular locations based on the uniprot database for 1373 proteins are shown in Table S2. The B/A ratios for proteins reported to be localized at apical or basolateral plasma membranes in the liver are presented (Figure 1). Significant differences in the B/A ratios were observed between 23 basolateral-localized (white column) and 7 both-localized (grey column) proteins ($p = 9.82 \times 10^{-7}$, Kolmogorov–Smirnov test), between 7 both-localized (grey column) and 11 apical-localized (black column) proteins ($p = 1.00 \times 10^{-4}$, Kolmogorov–Smirnov test), and between 23 basolateral-localized (white column) and 11 apical-localized (black column) proteins ($p = 6.99 \times 10^{-9}$, Kolmogorov–Smirnov test). Furthermore, the mean and variance of 7 both-localized proteins in Figure 1 were 1.04 and 0.0227, respectively (using 7 mean values but not using $7 \times 4$ (28 values)). The variances ($n = 4$) in B/A ratios were also calculated for each protein that is localized at basolateral (23 proteins) and apical (11 proteins) membranes. To show whether the B/A ratios of 23 basolateral- and 11 apical-localized proteins are statistically significantly different from distribution of both-localized protein group, the B/A ratios of individual basolateral and apical proteins were compared with the distribution of B/A ratios of 7 both-localized proteins by using a Student’s $t$-test followed by a Bonferroni correction. It showed that each B/A ratio for 23 basolateral- and 11 apical-localized proteins is significantly different from the distribution of B/A ratios of 7 both-localized proteins (Bonferroni-adjusted $p$ value < 0.05) (Figure 1). Based on the values of B/A ratio of 11 apical-, 7 both- and 23 basolateral-localized proteins, the mean and standard deviation of B/A ratio of each of the three groups were calculated and then the probability density was estimated by modeling the B/A ratios as log-normally distributed (Figure S1; the $p$-value for the Shapiro–Wilk test for normality on the logged B/A ratios were 0.959, 0.228 and 0.869 for apical-, both- and basolateral-localized groups, respectively). The intersection of the normal distributions of apical- and both-localized protein groups was 0.766. The intersection of the normal distributions for the basolateral- and both-localized protein groups was 1.42. These values would be used as border lines of B/A ratios to distinguish apical, both and basolateral localizations.
Figure 1. Comparison of the reported immunohistochemical localization and the basolateral/apical ratios that were comprehensively determined by SWATH analysis in this study. The basolateral/apical ratios of proteins whose membrane localizations were previously clarified by immunohistochemical analysis in liver are shown. The columns represent the mean ± SEM (n = 4). White column, proteins reported to be localized at the basolateral plasma membrane in liver; grey column, proteins reported to be localized at both basolateral and apical plasma membranes in the liver; black column, proteins reported to be localized at the apical plasma membrane in liver. Literature information concerning the membrane localization of individual molecules, Aqp9 [11], SLC2A2/Slc2a2 [12], Abcc6/Mrp6 [13], Slc26a1/Sat1 [14], Slc21a1/Oatp1a1 [13], Slc6a13/Gat3 [15], Slc10a1/Ntcp [13], Slc16a1/Mct1 [16], Abca1 [13], Slc16a7/Mct2 [17], Slc2a9/Glut9 [18], Na+/K+-Atpase β1 [19], Na+/K+-Atpase α1 [19], Atp11c [20], Slc21a10/Oatp1b2 [13], Slc22a1/Oct1 [13], Slc29a1/Ent1 [13], Slc23a1/Svt1 [21], Slc21a9/Oatp2b1 [13], Slc38a3/Snat3 [22], Slc22a5/Octn2 [23], Abcc3/Mrp3 [13], Na+/K+-Atpase β3 [19], Tubulins [24], Snap23 [25], Jcam1/Jam-A [26], Itgb1/Integrinβ-1 [27], Atp8b1 [13], Atp9a [28], Abcg5 [13], Abcc2/Mrp2 [13], Abcg8 [13], Abcb11/Bsep [13], Abcg2/Bcrp [13], Abcb4/Mdr2 [13], Alpl [29], Anpep [29] and Dpp4 [29]. Significant differences were observed between the basolateral-localized (white) and both-localized (grey) groups (p = 9.82 × 10−7, Kolmogorov–Smirnov test), between both-localized (grey) and apical-localized (black) groups (p = 1.00 × 10−4, Kolmogorov–Smirnov test), and between basolateral-localized (white) and apical-localized (black) groups (p = 6.99 × 10−9, Kolmogorov–Smirnov test). Furthermore, the mean and variance of 7 both-localized proteins were 1.04 and 0.0227, respectively (using 7 mean values but not using 7 × 4 (28 values)). The variances (n = 4) in B/A ratios were also calculated for each protein t is localized at basolateral (23 proteins) and apical (11 proteins) membranes. Using these values, a Student’s t-test followed by a Bonferroni correction showed that each B/A ratio for 23 basolateral- and 11 apical-localized proteins is significantly different from the distribution of B/A ratios of 7 both-localized proteins (Bonferroni-adjusted p value < 0.05). Furthermore, on the basis of probability density (Figure S1), the border lines of three groups were drawn at 0.766 and 1.42.
3.2. SWATH-Based Comprehensive Determinations of Basolateral and Apical Plasma Membrane Localizations in Mouse Liver

Based on the results of the validation described above, we can assume that molecules with a B/A ratio of less than 0.766 are localized to apical plasma membranes and molecules with a B/A ratio of more than 1.42 are localized to basolateral plasma membranes. To avoid the inclusion of molecules that are localized to organelle membranes in the cell, we focused on molecules that have been shown to be localized to cell membranes in the uniprot database (molecules including “cell membrane” as an uniprot subcellular location keyword). There are molecules, however, whose cell membrane localization is not registered in the uniprot database. Among the proteins not including “cell membrane” but including “membrane”, the proteins including the keywords organelle membrane other than “cell membrane” (e.g., mitochondrial membrane) were deleted, and the remaining proteins were selected as proteins that are potentially located in the plasma membrane. In other words, from the total 1373 proteins in Table S2, we selected molecules that that are localized to cell membranes and molecules that could potentially be localized to cell membranes, and the molecules with a B/A ratio of less than 0.766 (apical) or greater than 1.42 (basolateral) are listed in Tables 1 and 2, respectively. The B/A ratios of individual proteins listed in Tables 1 and 2 were compared with the distribution of B/A ratios of 7 both-localized proteins in Figure 1 by using a Student’s t-test followed by a Bonferroni correction. It showed that each B/A ratio for all the proteins is significantly different from the distribution of B/A ratios of 7 both-localized proteins (Bonferroni-adjusted p value < 0.05) (Table 1, Table 2, Tables S3 and S4).

It was estimated that 154 proteins are localized to the apical membrane of the mouse liver (Table 1). These included 17 receptors, 3 GPCR-related molecules, 11 CD antigens, 4 integrins, 2 adhesion molecules, 16 transporters, 2 channels, 6 pumps, 2 scaffold proteins, 1 proteoglycan, 42 enzymes, 3 Rho molecules, 9 Ras-related molecules, 32 other molecules and 4 uncharacterized molecules. Folr2 and slc46a1, which are involved in folate transport, were also included. A total of 125 proteins were estimated to be localized in the basolateral membrane of mouse liver cells (Table 2), including 11 receptors, 1 GPCR-related molecule, 2 CD antigens, 7 adhesion molecules, 34 transporters, 2 channels, 4 pumps, 10 scaffold proteins, 11 enzymes, 1 Rho molecule, 41 other molecules and 1 uncharacterized molecule. Scarb1, Asgr1 and Asgr2 were also included. A comparison of the data in Tables 1 and 2 shows that the number of membrane proteins, such as receptors, CD antigens and integrins, is much higher in apical membranes than in basolateral membranes. Similarly, the number of enzymes and Ras-related molecules was also much higher in the apical membrane. In contrast, there were considerably more adhesion molecules, scaffold proteins and transporters in basolateral membranes than in apical membranes.
Table 1. Functional classification of membrane proteins that are abundantly expressed in apical membrane fractions.

| Protein Name | Uniprot Accession Number | Plasma Membrane Localized? (Based on Uniprot) | B/A Ratio (Mean ± SEM) | Protein Name | Uniprot Accession Number | Plasma Membrane Localized? (Based on Uniprot) | B/A Ratio (Mean ± SEM) |
|--------------|--------------------------|-----------------------------------------------|-----------------------|--------------|--------------------------|-----------------------------------------------|-----------------------|
| **Receptors**|                          |                                               |                       | **Channels** |                          |                                               |                       |
| Folr2        | Q05685                   | Yes                                           | 0.367 ± 0.010         | Tmem63a      | Q91YT8                   | Yes                                           | 0.490 ± 0.013         |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Stra6l       | Q9DBN1                   | Yes                                           | 0.483 ± 0.002         |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1a1     | P50518                   | Yes                                           | 0.383 ± 0.010         |
| Cdc42        | P60763                   | Yes                                           | 0.501 ± 0.004         |              |                          |                                              |                       |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1b2     | P62814                   | Yes                                           | 0.513 ± 0.004         |
| **Pumps**    |                          |                                               |                       |              |                          |                                               |                       |
| Folr2        | Q05685                   | Yes                                           | 0.367 ± 0.010         | Tmem63a      | Q91YT8                   | Yes                                           | 0.490 ± 0.013         |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Stra6l       | Q9DBN1                   | Yes                                           | 0.483 ± 0.002         |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1a2     | P15920                   | Yes                                           | 0.690 ± 0.008         |
| **Rho**      |                          |                                               |                       |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1b2     | P62814                   | Yes                                           | 0.513 ± 0.004         |
| **Scaffold proteins** |                          |                                               |                       |              |                          |                                               |                       |
| Folr2        | Q05685                   | Yes                                           | 0.367 ± 0.010         | Tmem63a      | Q91YT8                   | Yes                                           | 0.490 ± 0.013         |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Stra6l       | Q9DBN1                   | Yes                                           | 0.483 ± 0.002         |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1a2     | P15920                   | Yes                                           | 0.690 ± 0.008         |
| **Proteoglycan** |                          |                                               |                       |              |                          |                                               |                       |
| Folr2        | Q05685                   | Yes                                           | 0.367 ± 0.010         | Tmem63a      | Q91YT8                   | Yes                                           | 0.490 ± 0.013         |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Stra6l       | Q9DBN1                   | Yes                                           | 0.483 ± 0.002         |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1a2     | P15920                   | Yes                                           | 0.690 ± 0.008         |
| **CD antigens** |                          |                                               |                       |              |                          |                                               |                       |
| Folr2        | Q05685                   | Yes                                           | 0.367 ± 0.010         | Tmem63a      | Q91YT8                   | Yes                                           | 0.490 ± 0.013         |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Stra6l       | Q9DBN1                   | Yes                                           | 0.483 ± 0.002         |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1a2     | P15920                   | Yes                                           | 0.690 ± 0.008         |
| **Others**   |                          |                                               |                       |              |                          |                                               |                       |
| Folr2        | Q05685                   | Yes                                           | 0.367 ± 0.010         | Tmem63a      | Q91YT8                   | Yes                                           | 0.490 ± 0.013         |
| Rpsa         | P14206                   | Yes                                           | 0.407 ± 0.001         | Aqp1         | Q02013                   | Yes                                           | 0.694 ± 0.004         |
| Stra6l       | Q9DBN1                   | Yes                                           | 0.483 ± 0.002         |              |                          |                                               |                       |
| Fcgr2        | P08101                   | Yes                                           | 0.483 ± 0.004         |              |                          |                                               |                       |
| Ptprc        | P06800                   | Yes                                           | 0.496 ± 0.003         | Atp6v1a2     | P15920                   | Yes                                           | 0.690 ± 0.008         |
| Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) |
|-------------|--------------------------|------------------------|-----------------------------------------------|-------------|--------------------------|------------------------|-----------------------------------------------|-------------|--------------------------|------------------------|-----------------------------------------------|
| Pecam1      | Q08481                   | 0.433 ± 0.005          | Yes                                           | Cemip2      | Q5FW13                   | 0.438 ± 0.002          | Yes                                           | Clec4g      | Q8BNX1                   | 0.590 ± 0.002          | Yes                                           |
| Cd1d1       | P11609                   | 0.511 ± 0.005          | Yes                                           | Got2        | P05202                   | 0.444 ± 0.002          | Yes                                           | Irgm1       | Q60766                   | 0.599 ± 0.002          | Yes                                           |
| Bst2        | Q8R2Q8                   | 0.563 ± 0.002          | Yes                                           | Abhd17b     | Q7M759                   | 0.450 ± 0.020          | Yes                                           | Stxs8       | O89893                   | 0.604 ± 0.003          | Potentially                                      |
| Cd36        | Q08857                   | 0.626 ± 0.006          | Yes                                           | Alpl        | P09242                   | 0.454 ± 0.004          | Yes                                           | Fam234b     | Q8BY18                   | 0.640 ± 0.018          | Potentially                                      |
| Cd47        | Q61735                   | 0.686 ± 0.003          | Yes                                           | Nos3        | P70313                   | 0.475 ± 0.007          | Yes                                           | Attraid     | Q6PGD0                   | 0.657 ± 0.034          | Yes                                           |
| Cd59a       | O55186                   | 0.709 ± 0.013          | Yes                                           | Enpp4       | Q8BTJ4                   | 0.490 ± 0.005          | Yes                                           | Pttg1ip     | Q8R143                   | 0.657 ± 0.009          | Yes                                           |
| Cd68        | P31996                   | 0.760 ± 0.025          | Yes                                           | P4hb        | P09103                   | 0.510 ± 0.002          | Yes                                           | Gdi2        | Q61598                   | 0.661 ± 0.010          | Potentially                                      |
| Integrins   |                          |                        |                                               |             |                          |                        |                                               |             |                          |                        |                                               |
| Itga9       | B8JK39                   | 0.399 ± 0.014          | Potentially                                    | Pdia6       | Q922R8                   | 0.533 ± 0.001          | Yes                                           | Clec2d      | Q91V08                   | 0.704 ± 0.008          | Yes                                           |
| Itga1       | Q3V3R4                   | 0.622 ± 0.002          | Potentially                                    | Ece1        | Q4PZA2                   | 0.536 ± 0.002          | Yes                                           | Tmed1       | Q3V099                   | 0.717 ± 0.033          | Yes                                           |
| Itgal       | P24063                   | 0.648 ± 0.025          | Yes                                           | Adam23      | Q9R1V7                   | 0.552 ± 0.039          | Yes                                           | Mal2        | Q8B508                   | 0.724 ± 0.002          | Yes                                           |
| Itgav       | P43406                   | 0.707 ± 0.003          | Yes                                           | Naalad2     | Q9CZR2                   | 0.557 ± 0.008          | Yes                                           | Kct2        | Q8K201                   | 0.730 ± 0.011          | Potentially                                      |
| Adhesion molecules |              |                        |                                               |             |                          |                        |                                               |             |                          |                        |                                               |
| Esam        | Q925F2                   | 0.615 ± 0.030          | Yes                                           | Mgl1        | O35678                   | 0.590 ± 0.005          | Potentially                                    | Hpcal1      | P62748                   | 0.750 ± 0.017          | Potentially                                      |
| Icam2       | P35330                   | 0.701 ± 0.010          | Potentially                                    | Cgt6        | Q6PDE7                   | 0.610 ± 0.011          | Potentially                                    | Lrc57       | Q9D1G5                   | 0.750 ± 0.018          | Potentially                                      |
| Transporters |                      |                        |                                               | Ctsb        | Q6PDE7                   | 0.610 ± 0.011          | Potentially                                    | Tmem123     | Q91Z22                   | 0.750 ± 0.008          | Potentially                                      |
| Slc46a3     | Q9DC26                   | 0.390 ± 0.004          | Potentially                                    | Fr5S1       | Q8K385                   | 0.640 ± 0.008          | Potentially                                    | Rpl2        | Q9EPK2                   | 0.765 ± 0.004          | Yes                                           |
| Slc39a4     | Q78IQ7                   | 0.414 ± 0.015          | Yes                                           | Cdp         | O89001                   | 0.649 ± 0.011          | Yes                                           |                     |                          |                        |                                               |
| Slc44a2     | Q8BY89                   | 0.449 ± 0.009          | Potentially                                    | B4gatL1     | P15535                   | 0.650 ± 0.005          | Yes                                           |                     |                          |                        |                                               |
| Abcb4       | P21440                   | 0.464 ± 0.001          | Yes                                           | Hpd         | P49429                   | 0.668 ± 0.001          | Potentially                                    | Tmem59      | Q9QY73                   | 0.291 ± 0.009          | Yes                                           |
| Abcg2       | Q7TM55                   | 0.483 ± 0.001          | Yes                                           | ClgatL1     | Q9J106                   | 0.675 ± 0.009          | Potentially                                    | Tmem176a    | Q9DCS1                   | 0.363 ± 0.009          | Potentially                                      |
| Abcb11      | Q9QY30                   | 0.492 ± 0.000          | Yes                                           | Adam10      | O35598                   | 0.683 ± 0.003          | Yes                                           | Tm9sf4      | Q8BH24                   | 0.578 ± 0.002          | Potentially                                      |
| Abcg8       | Q9DBM0                   | 0.520 ± 0.007          | Yes                                           | Eno1        | P17182                   | 0.684 ± 0.003          | Yes                                           | Tm7sf3      | Q9CRG1                   | 0.662 ± 0.012          | Yes                                           |
| Sla2a8      | Q9JIF3                   | 0.524 ± 0.017          | Yes                                           | Lnpep       | Q8C129                   | 0.690 ± 0.005          | Yes                                           |                     |                          |                        |                                               |
| Sla46a1     | Q6PEM8                   | 0.530 ± 0.005          | Yes                                           | Park7       | Q9L9X0                   | 0.702 ± 0.006          | Yes                                           |                     |                          |                        |                                               |
| Abcb6       | Q9DC29                   | 0.532 ± 0.001          | Yes                                           | P4k2b       | Q8CBDQ5                  | 0.723 ± 0.023          | Potentially                                    |                     |                          |                        |                                               |
| Abcc2       | Q8VI47                   | 0.564 ± 0.001          | Yes                                           | P1k3r4      | Q8VD65                   | 0.740 ± 0.033          | Potentially                                    |                     |                          |                        |                                               |

**Table 1. Cont.**
Table 1. Cont.

| Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) |
|--------------|--------------------------|------------------------|-----------------------------------------------|--------------|--------------------------|------------------------|-----------------------------------------------|--------------|--------------------------|------------------------|-----------------------------------------------|
| Slc38a7      | Q8BWH0                   | 0.591 ± 0.006          | Potentially                                   | Nedd4        | P46935                   | 0.741 ± 0.005          | Yes                                           |                           |                           |                          |                                |
| Abcg5        | Q99PE8                   | 0.596 ± 0.011          | Yes                                           | Cnp          | P16330                   | 0.754 ± 0.012          | Potentially                                   |                           |                           |                          |                                |
| Slc12a9      | Q99MR3                   | 0.635 ± 0.007          | Yes                                           | Tgm2         | P21981                   | 0.765 ± 0.019          | Yes                                           |                           |                           |                          |                                |
| Slc10a5      | Q5PT54                   | 0.704 ± 0.038          | Potentially                                   |                           |                           |                          |                                |
| Slc30a10     | Q3UVU3                   | 0.750 ± 0.027          | Yes                                           |                           |                           |                          |                                |

Among the total 1373 proteins that were quantified (Table S2), proteins whose basolateral/apical (B/A) ratios were less than 0.766 were selected. Furthermore, proteins including the term “cell membrane” in the uniport subcellular location information were selected. On the other hand, among proteins not including “cell membrane” but including “membrane”, the proteins that included the keywords of organelle membranes other than cell membrane (e.g., mitochondrial membrane) were deleted, and the remaining proteins were selected as proteins that could be potentially located on the plasma membrane. The list of “cell membrane” proteins above was combined with that of proteins that could be potentially located on the plasma membrane. B/A ratio represents the mean ± SEM (n = 4). Furthermore, the mean and variance of 7 both-localized proteins in Figure 1 were 1.04 and 0.0227, respectively. The variances of the B/A ratios were also calculated for each protein in this table. Using these values, a Student’s t-test followed by a Bonferroni correction showed that each B/A ratio for all the proteins listed in this table is significantly different from the distribution of B/A ratios of 7 both-localized proteins (Bonferroni-adjusted p value < 0.05). The p values are listed in Table S3.

Table 2. Functional classification of membrane proteins that are abundantly expressed in basolateral membrane fractions.

| Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) |
|--------------|--------------------------|------------------------|-----------------------------------------------|--------------|--------------------------|------------------------|-----------------------------------------------|--------------|--------------------------|------------------------|-----------------------------------------------|
| Scarb1       | Q61009                   | 2.78 ± 0.02            | Yes                                           | Slc4a1       | P04919                   | 5.73 ± 0.06            | Yes                                           | Arhgef12     | Q8R4H12                  | 1.43 ± 0.05            | Potentially                                  |
| Ptprf        | A2A8L5                   | 1.98 ± 0.01            | Potentially                                   | Pdzk1        | Q9JL4                    | 5.28 ± 0.01            | Yes                                           |                           |                           |                          |                                |
| Adra1b       | P97717                   | 1.95 ± 0.03            | Yes                                           | Slc9a3r1     | P70441                   | 4.74 ± 0.03            | Yes                                           |                           |                           |                          |                                |
| Ptprg        | Q05909                   | 1.91 ± 0.07            | Potentially                                   | Slc2a2       | P14246                   | 3.19 ± 0.02            | Yes                                           | Actn1        | Q7TPR4                   | 5.68 ± 0.14            | Yes                                           |
| Lsr          | Q99KG5                   | 1.90 ± 0.01            | Yes                                           | Abca8a       | Q8K442                   | 3.09 ± 0.02            | Yes                                           | Utrn         | E9Q6R7                   | 5.31 ± 0.06            | Yes                                           |
| Ptprd        | Q64487                   | 1.64 ± 0.08            | Potentially                                   | Abcc6        | Q9R157                   | 3.02 ± 0.01            | Yes                                           | Std10        | Q9JMD3                   | 4.83 ± 0.39            | Potentially                                  |
| Egfr         | Q01279                   | 1.62 ± 0.00            | Yes                                           | Slc26a1      | P58735                   | 2.93 ± 0.01            | Yes                                           | Tspan4       | Q9DCK3                   | 4.72 ± 0.14            | Potentially                                  |
| Insr         | P15208                   | 1.52 ± 0.01            | Yes                                           | Slc1a2       | P43006                   | 2.92 ± 0.04            | Yes                                           | Pascin3      | Q9JRF8                   | 4.32 ± 0.04            | Yes                                           |
| Erbb3        | Q61526                   | 1.45 ± 0.04            | Potentially                                   | Slc1a1       | Q9QXZ6                   | 2.87 ± 0.01            | Yes                                           | Cask         | O70589                   | 4.27 ± 0.03            | Yes                                           |
| Asgr1        | P34927                   | 1.43 ± 0.00            | Potentially                                   | Slc6a6       | O35316                   | 2.75 ± 0.03            | Yes                                           | Lima1        | Q9ERG0                   | 4.19 ± 0.03            | Yes                                           |
| Asgr2        | P24721                   | 1.43 ± 0.00            | Potentially                                   | Slc6a13      | P31649                   | 2.75 ± 0.04            | Yes                                           | Sntb1        | Q99L88                   | 3.81 ± 0.04            | Yes                                           |
| Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) |
|-------------|-------------------------|------------------------|-----------------------------------------------|-------------|-------------------------|------------------------|-----------------------------------------------|
| **GPCR related molecule** |             |                        |                                               |             |                        |                        |                                               |
| Gna12       | P27600                  | 1.66 ± 0.05            | Yes                                           | Slc6a11     | P31650                  | 2.75 ± 0.03            | Potentially Scrib                        |
|             |                         |                        |                                               | Slc10a1     | O08705                  | 2.70 ± 0.01            | Potentially C2cd2                        |
| Bsg         | P18572                  | 2.50 ± 0.01            | Yes                                           | Abca1       | P41233                  | 2.66 ± 0.01            | Yes                                          |
| Cd82        | P40237                  | 2.37 ± 0.01            | Yes                                           | Slc10a1     | Q07451                  | 2.63 ± 0.09            | Yes                                          |
| **CD antigens** |             |                        |                                               |             |                        |                        |                                               |
|             |                         |                        |                                               | Slc10a1     | Q3T9X0                  | 2.50 ± 0.01            | Yes                                          |
|             |                         |                        |                                               | Slc16a1     | Q88343                  | 2.42 ± 0.03            | Yes                                          |
|             |                         |                        |                                               | Abca8b      | Q8K440                  | 2.32 ± 0.01            | Yes                                          |
| **Adhesion molecules** |             |                        |                                               |             |                        |                        |                                               |
| Ctnnd1      | P30999                  | 2.21 ± 0.00            | Yes                                           | Slc6a11     | Q07451                  | 2.69 ± 0.01            | Yes                                          |
| Ctnnb1      | Q02248                  | 2.20 ± 0.01            | Yes                                           | Slc16a1     | Q88343                  | 2.42 ± 0.03            | Yes                                          |
| Ctnna1      | P26231                  | 2.01 ± 0.01            | Yes                                           | Abca8b      | Q8K440                  | 2.32 ± 0.01            | Yes                                          |
| Cd82        | P15116                  | 1.93 ± 0.02            | Yes                                           | Slc16a1     | Q07451                  | 2.63 ± 0.09            | Yes                                          |
| Clnd1       | Q9CQX5                  | 1.75 ± 0.06            | Potentially                                   | Slc2a9      | Q9J7M1                  | 2.11 ± 0.01            | Yes                                          |
| Cad1        | Q8KS58                  | 1.74 ± 0.05            | Yes                                           | Slc3a14     | Q9J7M1                  | 2.11 ± 0.01            | Yes                                          |
| Clnd3       | Q9ZOG9                  | 1.64 ± 0.02            | Yes                                           | Slc2a9      | Q9J7M1                  | 2.11 ± 0.01            | Yes                                          |
| **Scaffold proteins** |             |                        |                                               |             |                        |                        |                                               |
| Rdx         | P26043                  | 2.72 ± 0.02            | Yes                                           | Slc3a14     | Q9J7M1                  | 2.11 ± 0.01            | Yes                                          |
| Ap2b1       | Q9DBGC3                 | 2.05 ± 0.01            | Yes                                           | Slc4a11     | Q6X893                  | 1.82 ± 0.02            | Yes                                          |
| Ezr         | P26040                  | 1.98 ± 0.04            | Yes                                           | Slc2b1      | Q8 getInstance()        | 1.94 ± 0.01            | Yes                                          |
| Picalm      | Q7MV63                  | 1.94 ± 0.02            | Yes                                           | Slc2a1      | Q17809                  | 1.59 ± 0.05            | Yes                                          |
| Dnm2        | P39054                  | 1.74 ± 0.02            | Potentially                                   | Slc4a11     | Q6X893                  | 1.82 ± 0.02            | Yes                                          |
| Ap2m1       | P84091                  | 1.68 ± 0.01            | Yes                                           | Slc4a11     | Q6X893                  | 1.82 ± 0.02            | Yes                                          |
| Ap2a2       | P17427                  | 1.66 ± 0.00            | Yes                                           | Slc4a11     | Q6X893                  | 1.82 ± 0.02            | Yes                                          |
| **Enzymes** |             |                        |                                               |             |                        |                        |                                               |
| Ap2s1       | P62743                  | 1.65 ± 0.02            | Yes                                           | Slc4a11     | Q6X893                  | 1.82 ± 0.02            | Yes                                          |
| Clin1       | Q99KN9                  | 1.62 ± 0.01            | Potentially                                   | Slc3a14     | Q9J7M1                  | 2.11 ± 0.01            | Yes                                          |
| Ap2a1       | P17426                  | 1.60 ± 0.01            | Yes                                           | Slc3a14     | Q9J7M1                  | 2.11 ± 0.01            | Yes                                          |

**Cont.**
Table 2. Cont.

| Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) | Protein Name | Uniprot Accession Number | B/A Ratio (Mean ± SEM) | Plasma Membrane Localized? (Based on Uniprot) |
|--------------|--------------------------|------------------------|-----------------------------------------------|--------------|--------------------------|------------------------|-----------------------------------------------|--------------|--------------------------|------------------------|-----------------------------------------------|
| **Channels** |                          |                        |                                               |              |                          |                        |                                               |              |                          |                        |                                               |
| Aqp9         | Q9JJ3                    | 3.72 ± 0.02            | Yes                                           | Atp5f1a      | Q03265                   | 1.86 ± 0.00            | Yes                                           | Lrm4         | P59383                   | 1.61 ± 0.09            | Potentially          |
| Clic4        | Q9QYB1                   | 2.94 ± 0.03            | Yes                                           | Steap4       | Q92386                   | 1.76 ± 0.01            | Yes                                           | Stxbp3       | Q60770                   | 1.61 ± 0.01            | Yes                              |
|              |                          |                        |                                               | Ilk          | O55222                   | 1.75 ± 0.03            | Yes                                           | Ndrg1        | Q62433                   | 1.50 ± 0.02            | Yes                              |
|              |                          |                        |                                               |              |                          |                        |                                               | Eps15        | P42567                   | 1.43 ± 0.02            | Yes                              |
| **Pumps**    |                          |                        |                                               |              |                          |                        |                                               |              |                          |                        |                                               |
| Fxyd1        | Q9Z239                   | 2.45 ± 0.02            | Yes                                           | Pik3c2a      | Q61194                   | 1.59 ± 0.07            | Yes                                           |              |                          |                        | Uncharacterized molecules |
| Atp1b1       | P14094                   | 2.36 ± 0.00            | Yes                                           | Adam17       | Q9Z0F8                   | 1.55 ± 0.04            | Yes                                           | Tmem150a     | Q91WN2                   | 3.01 ± 0.10            | Yes                              |
| Atp1a1       | Q8VDN2                   | 2.23 ± 0.00            | Yes                                           |              |                          |                        |                                               |              |                          |                        |                                               |
| Atp1b3       | P97370                   | 1.69 ± 0.01            | Yes                                           |              |                          |                        |                                               |              |                          |                        |                                               |

Among the total 1373 proteins that were quantified (Table S2), the proteins whose basolateral/apical (B/A) ratios were more than 1.42 were selected. In addition, the proteins including “cell membrane” in uniport subcellular location information were selected. On the other hand, among proteins not including “cell membrane”, but including “membrane”, proteins including the keywords of organelle membrane other than cell membrane (e.g., mitochondrial membrane) were deleted, and the remaining proteins were selected as proteins that could be potentially located on the plasma membrane. The list of “cell membrane” proteins above was combined with that of proteins potentially located in plasma membrane. B/A ratio represents the mean ± SEM (n = 4). Furthermore, the mean and variance of 7 both-localized proteins in Figure 1 were 1.04 and 0.0227, respectively. The variances in B/A ratios were also calculated for each protein in this table. Using these values, a Student’s t-test followed by a Bonferroni correction showed that each B/A ratio for all the proteins listed in this table is significantly different from the distribution of B/A ratios of 7 both-localized proteins (Bonferroni-adjusted p value < 0.05). The p values are listed in Table S4.
4. Discussion

In this study, we report on the development of a method for determining the apical and basolateral localization of membrane proteins in a comprehensive manner that does not involve the use of antibodies. In this study, we evaluated the accuracy of the SWATH method using 23, 11 and 7 proteins, which have been reported to be localized to the apical, the basolateral and both plasma membranes of hepatocytes, respectively, as model molecules. The results showed that apical and basolateral membrane proteins could be clearly distinguished (Figure 1). Thus, the method proved to be highly accurate and comprehensive in determining the apical and basolateral localization of membrane proteins.

The list of molecules shown in Tables 1 and 2 will be useful in terms of drug delivery. The selective delivery of drugs to the liver is important in the treatment of liver diseases. The targeted delivery of oligonucleotides to liver hepatocytes using N-acetylgalactosamine (GalNAc) conjugates that bind to the asialoglycoprotein receptor (Asgr) has become a breakthrough approach in the field of therapeutic oligonucleotides [1]. Although it is known that Asgr is localized on basolateral membranes, in this study, we were able to separately quantify Asgr1 and Asgr2, and the results clearly shows that both molecules are localized on basolateral membranes (Table 2). Regarding Asgr, it has been reported that Asgr-mediated delivery can become saturated [30]. As shown in Table 2, not only Asgr1 and Asgr2, but also the Scarb1, Ptprf, Adra1b, Ptprg, Lsr, Lsr, Ptprd, Egfr, Insr and Erbb3 receptors are localized to the basolateral membrane. Not only receptors, but also CD antigens, integrins, transporters and other membrane proteins can be internalized by the binding of ligands such as antibodies. Therefore, it is hoped that this list will be of use in terms of drug delivery for liver diseases. Several types of membrane protein internalization mechanisms are known, including clathrin-mediated and caveolin-mediated types. In the basolateral membrane of the liver, many molecules that are involved in clathrin-mediated endocytosis, such as the AP-2 complex (Ap2b1, Ap2m1, Ap2a2, Ap2s1 and Ap2a1), Picalm, Dnm2 and Clint1 were localized. Therefore, among the molecules that are localized to basolateral membranes, those that internalize in a clathrin-dependent manner may be useful for the smooth efficient delivery of drugs to the liver. In contrast, the localization of caveolin-1 in apical membranes (Table 1) suggests that caveolin-mediated internalization may be more active in apical membranes.

In contrast, most drugs (which can be toxic) tend to accumulate in the liver. Therefore, it is important to establish a drug delivery system that does not transfer drugs to normal hepatocytes, but, rather, to diseased tissue. In normal hepatocytes, DDS products in blood cannot access the membrane proteins of the apical membrane because of the presence of tight junctions. Therefore, membrane proteins that are localized to the apical membrane in normal hepatocytes (Table 1) may be promising receptors for DDS systems that avoid hepatotoxicity. Folr2 and slc46a1, which prefer folate as a ligand or substrate, have been shown to become localized to the apical membrane (Table 1). The binding of folic acid to the surface of liposomes has been reported to decrease the number of liposomes that are transferred to the liver and, therefore, to increase the amount transferred to cancerous tissues [2]. This suggests that when considering active targeting to tissues other than the liver, targeting membrane proteins that are present on the surface of the relevant tissue but are not localized to the basolateral membrane of the liver may increase the amount transferred to the target tissue and decrease the amount transferred to the liver.

Interestingly, many of the low molecular weight G-protein Rab molecules that are involved in epithelial polarity transport were found to be localized to the apical membrane (Table 1; Rab1A, Rab5a, Rab5b, Rab5c, Rab8a, Rab9a, Rab18 and Rab35). In general, Rab molecules are involved in intracellular vesicular trafficking to the apical membranes, where they support the localization of membrane proteins to the apical membrane. Although the Rab subtypes in hepatocytes have not been fully elucidated, the findings reported in this study indicate that the above Rab subtypes are localized to the apical membrane. In contrast, no Rab molecules were detected in the basolateral membrane fraction (Table 2). It will be interesting to determine whether these Rab subtypes are involved in vesicular
trafficking to the apical membrane in hepatocytes. However, further research would be needed in the future to validate the estimated localizations listed in Tables 1 and 2 by immunohistochemistry.

Researching DDSs that deliver cargoes into the central nervous systems (CNS), such as the brain and spinal cord, is an important issue. Given the fact that the blood–brain barrier and the blood–arachnoid barrier are large surface area barriers, it would be desirable for DDS products to pass through these barriers. Methods for separating the blood- and CNS-side plasma membranes of barrier cells have been reported. Briefly, after the isolation of blood vessels or leptomeninges from the brain or spinal cord, the blood- and CNS-side plasma membranes can be fractionated by density gradient centrifugation using different concentrations of ficoll or sucrose [4,31]. It is also important to list molecules that are not localized to the blood-side plasma membranes of the intestinal and renal epithelial cells to avoid adverse effects due to transfer to these organs, which have a high blood flow. Methods for fractionating the blood- and luminal-side plasma membranes of each epithelial cell have been established [32–35]. By combining the cell membrane fractionation methods for each of these tissues with the SWATH method, an exhaustive list of molecules that are localized or are not localized to the blood-side plasma membrane can be generated with a high degree of accuracy and comprehensiveness.

5. Conclusions

By combining the conventional apical/basolateral membrane separation method with high precision SWATH proteomics, it was possible to comprehensively determine the localization of apical and basolateral membrane proteins (Figures 1 and 2). In terms of drug delivery, it is hoped that this method will be found to be applicable to other organs and human tissues in the future, so that a list of proteins that are localized on membranes of all organs can be established.

![Figure 2](Image)

Figure 2. Schematic illustration of the apical and basolateral localization of proteins in mouse hepatocyte determined in this study. The apical and basolateral localization of proteins are illustrated based on the data of Tables 1 and 2, respectively. In physiologically normal conditions, the drug delivery systems administered into the systemic circulation usually access the basolateral surface of hepatocytes. Therefore, the membrane proteins that are localized at the basolateral membrane potentially support the cellular uptake drugs from drug delivery systems.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biomedicines10020383/s1. Figure S1: Probability distribution based on 11 apical-, 7 both- and 23 basolateral-localized proteins. Table S1: In silico peptide selection criteria. Table S2: Ratios of the protein expression levels of all the proteins quantified by SWATH analysis between basolateral and apical membrane fractions in mouse liver. Table S3: The p values of the proteins listed in Table 1. Table S4: The p values of the proteins listed in Table 2.

Author Contributions: Conceptualization, Y.U.; methodology, S.H., R.G. and Y.U.; software, S.H., R.G. and Y.U.; validation, S.H., R.G. and Y.U.; formal analysis, S.H., R.G. and Y.U.; investigation, S.H., R.G. and Y.U.; resources, S.H., R.G. and Y.U.; data curation, S.H., R.G. and Y.U.; writing—original draft preparation, Y.U.; writing—review and editing, Y.U.; visualization, S.H. and Y.U.; supervision, Y.U.; project administration, Y.U.; funding acquisition, Y.U. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal experiments were conducted based on ARRIVE guidelines, and the protocol was approved by the Institutional Animal Care and Use Committee at Tohoku University (protocol code 2018PhA-028-01, approved on 1 September 2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw MS data are available on the Peptide Atlas webpage with Identifier PASS01726.

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Conflicts of Interest: The authors declare no conflict of interest.

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