A Multiplexed Quantitative Strategy for Membrane Proteomics

OPPORTUNITIES FOR MINING THERAPEUTIC TARGETS FOR AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

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Toward multiplexed, comprehensive, and robust quantification of the membrane proteome, we report a strategy combining gel-assisted digestion, iTRAQ (isobaric tags for relative and absolute quantitation) labeling, and LC-MS/MS. Quantitation of four independently purified membrane fractions from HeLa cells gave high accuracy (<8% error) and precision (<12% relative S.D.), demonstrating a high degree of consistency and reproducibility of this quantitation platform. Under stringent identification criteria (false discovery rate = 0%), the strategy efficiently quantified membrane proteins; as many as 520 proteins (91%) were membrane proteins, each quantified based on an average of 14.1 peptides per integral membrane protein. In addition to significant improvements in signal intensity for most quantified proteins, most remarkably, topological analysis revealed that the biggest improvement was achieved in detection of transmembrane peptides from integral membrane proteins with up to 19 transmembrane helices. To the best of our knowledge, this level of coverage exceeds that achieved previously using MS and provides superior quantitation accuracy compared with other methods. We applied this approach to the first proteomics delineation of phenotypic expression in a mouse model of autosomal dominant polycystic kidney disease (ADPKD). By characterizing kidney cell plasma membrane from wild-type versus PKD1 knock-out mice, 791 proteins were quantified, and 67 and 37 proteins showed >2-fold up-regulation and down-regulation, respectively. Some of these differentially expressed membrane proteins are involved in the mechanisms underlying major abnormalities in ADPKD, including epithelial cell proliferation and apoptosis, cell-cell and cell-matrix interactions, ion and fluid secretion, and membrane protein polarity. Among these proteins, targeting therapeutics to certain transporters/receptors, such as epidermal growth factor receptor, has proven effective in preclinical studies of ADPKD; others are known drug targets in various diseases. Our method demonstrates how comparative membrane proteomics can provide insight into the molecular mechanisms underlying ADPKD and the identification of potential drug targets, which may lead to new therapeutic opportunities to prevent or retard the disease. Molecular & Cellular Proteomics 7: 1983–1997, 2008.

Many membrane proteins are implicated in particular disease states and often are attractive therapeutic targets (1). Comprehensive and quantitative profiles of membrane proteins facilitate our understanding of their roles in regulating biological processes and in cellular signaling. However, the analysis of membrane proteins is experimentally challenging because of their hydrophobic nature and low abundance, which seriously complicate their solubilization, sample handling, preparation, separation, and analysis (2–5). Although ~20–30% of open reading frames of most sequenced genomes are estimated to encode integral membrane proteins (6), the membrane proteome has not been mapped as comprehensively as the soluble proteome (2, 3).

For membrane proteomics studies, conventional strategies using two-dimensional gel electrophoresis (2DE)¹ have had some success in profiling the bacterial outer membrane (7), organelle membranes (8), and plasma membrane (9). However, the serious under-representation of membrane proteins reported in the above studies reveals the drawback of low

¹ The abbreviations used are: 2DE, two-dimensional gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantitation; SCX, strong cation exchange; TMHMM, transmembrane hidden Markov model; TMH, transmembrane helix; ADPKD, autosomal dominant polycystic kidney disease; Na⁺/K⁺-ATPase α1 and β1, sodium/potassium-transporting ATPase α1 and β1 chain; SILAC, stable isotope labeling by amino acids in cell culture; BALF, bronchoalveolar lavage fluid; APS, ammonium persulfate; TEMED, N,N,N’,N’-tetramethylethlenediamine; TEABC, triethylammonium bicarbonate; ABC, ammonium bicarbonate; MMTS, methyl methanethiosulfonate; FA, formic acid; IPI, International Protein Index; R.S.D., relative standard deviation; WT, wild type; EGFR, epidermal growth factor receptor; COX, cyclooxygenase; PKD, polycystic kidney disease; R, ratio.
sensitivity. To alleviate the drawbacks of 2DE, stable isotopic labeling in conjunction with off-line or on-line multidimensional LC-MS/MS analysis offers greater sensitivity. The two most common ways of incorporating stable isotopes into biological samples are through either ICAT or metabolic stable isotope labeling by amino acids in cell culture (SILAC) (10). However, many proteins, especially membrane proteins, have few (if any) cysteine residues, resulting in smaller numbers of quantifiable proteins in ICAT. Furthermore the high concentration (>0.05%) of denaturing reagents (e.g. SDS) required for ICAT labeling, especially for membrane proteins, is incompatible with ICAT alkylation of cysteine residues because of reduced accessibility after formation of the SDS-protein complex. Although an optimized strategy using a smaller ICAT analog was recently reported by Ramus et al. (11), the quantification efficiency of this labeling strategy on the proteome scale remains to be evaluated. Alternatively SILAC offers more options for selection of isotopically labeled amino acids such as arginine, leucine, lysine, serine, methionine, and tyrosine (12). Although some successes have been reported in analysis of the membrane proteome (13, 14), the major drawback of SILAC is that it is most applicable to cell culture rather than tissue or body fluids.

Recently a new amine labeling strategy at the peptide level using isobaric tags (iTRAQ) was developed for multiplexed protein quantitation (15). In contrast to other quantitative methods based on MS intensity of the isotopically paired peptides, the relative intensity of signature reporter ions of $m/z$ 114, 115, 116 and 117 in an MS/MS spectrum is used to quantify protein levels. Since its development, it has become popular because of the following advantages. First, the multiplexing reagents target the N terminus as well as the lysine side chains of peptides, and thus each peptide fragment can be labeled with better efficiency compared with protein-level labeling. Second, the intensity of both the precursor ion and resultant MS/MS fragments is greatly enhanced by summing of four isobarically iTRAQ-labeled sample sets, thereby increasing the number of peptides identified and quantified and thus increasing the quantification accuracy. The iTRAQ strategy has been widely applied for comparative analysis characterization of the soluble proteome (16, 17), particularly for enriched subproteomes (18). Whether it can offer enhanced identification confidence and quantitation accuracy for the membrane proteome will require further evaluation.

Although shotgun proteomics for membrane proteins produces more satisfactory results than 2DE, the incompatibility of high concentrations of detergent/solvent with subsequent enzymatic digestion and LC-MS/MS analysis still presents substantial limitations. Proteolysis of integral membrane proteins usually results in low sequence coverage because their low aqueous solubility and high hydrophobicity render them relatively inaccessible to proteases. Several groups have attempted to circumvent this limitation by using organic solvents (2), organic acids (19), microwave-assisted acid hydrolysis (20), or detergent-containing aqueous solution (3). Recent studies have shown that SDS concentrations up to 1% are compatible with in-solution digestion for membrane proteins (21, 22). However, the use of SDS reduces resolution during reverse-phase LC and suppresses peptide ionization efficiency. Alternative digestion using methanol-assisted protein solubilization has been effective for detecting integral membrane proteins (22, 23). Although methanol (up to 60%) is easily removed after proteolysis, both SDS and methanol, even at low levels, substantially reduce protease activity. For example, the activity of trypsin (the most commonly used protease) falls to ~20, 1, and 30% in the presence of 0.1% SDS, 0.5% SDS, and 50% methanol, respectively.

Recently Lu and Zhu (24) described a Tube-Gel digestion protocol that incorporates protein into a polyacrylamide gel matrix in a glass tube without electrophoresis. The digestion efficiency is surprisingly good: a single 2.5-h LC-MS/MS analysis using Tube-Gel digestion yields results comparable to 20 LC-MS/MS analyses from SDS-PAGE fractionation (24). Inspired by Tube-Gel digestion, we reported a gel-assisted digestion that is more compatible with high concentrations of a variety of detergents and allows more efficient removal of detergent (25). The method was applied to the first global description of the bronchoalveolar lavage fluid (BALF) proteome in patients with ventilator-associated pneumonia (25). It effectively overcame interference by the intrinsic high salt concentration in BALF, and the 206 identified proteins represent the most comprehensive proteome map of BALF. Thus, with good digestion efficiency, gel-assisted digestion may be ideal for peptide-level labeling, such as iTRAQ, with certain protocol modifications to ensure compatibility in the labeling reaction. In this study, we report a strategy integrating enhanced gel-assisted digestion and iTRAQ labeling for multiplex quantitative profiling of the membrane proteome. Specifically we aimed to achieve 1) higher identification/quantification numbers, 2) enhanced quantification for integral membrane proteins, and 3) broad compatibility with a variety of solubilization reagents with robust applicability to diverse biological systems. First we evaluated the reproducibility of gel-assisted digestion and its compatibility with iTRAQ labeling for multiplex quantitation in comparison with the most common in-solution or Tube-Gel digestion techniques. Performance assessment, including coverage of quantified peptides and proteins, quantitation accuracy, and precision, was evaluated via large scale quantitation of replicate membrane fractions from HeLa cells. Finally the new approach was applied to analyze the membrane proteome for the mouse model of autosomal dominant polycystic kidney disease (ADPKD).

ADPKD is the most prevalent and potentially lethal inherited human renal disease (26). However, current treatments are mostly limited to renal replacement therapy by dialysis, which extends survival by an average of 7 years, or by transplanta-

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2 Data from Waters Corp., Library Number 720000468EN.
tion coupled with aggressive management of hypertension (27). Although genetic manifestations of the disease have revealed that a mutation in the gene PKD1 contributes the major etiology of the disease (28), most of the efforts to date have focused on PKD1 genetic heterogeneity and the potential contribution of modifier genes (29). Systematic mapping of phenotypic expression of the disease and uncovering perturbed cellular networks remain to be further investigated, and to our knowledge no large scale proteomics or genomics studies have been reported. Here we present the first comparative proteomics study of the plasma membrane from the kidney of control and PKD1 knock-out mice. We identified many differentially expressed membrane proteins in the knock-out mouse that may provide insight into the molecular mechanism underlying the development and progression of ADPKD as well as an opportunity for the development of a more effective pathophysiology-based therapy.

**EXPERIMENTAL PROCEDURES**

Materials—Monomeric acrylamide/bisacrylamide solution (40%, 29:1) was purchased from Bio-Rad. Trypsin (modified, sequencing grade) was obtained from Promega (Madison, WI). The BCA and Bradford protein assay reagent kits were obtained from Pierce. SDS was purchased from GE Healthcare. Ammonium persulfate (APS), iodoacetamide, and TEMED were purchased from Amersham Biosciences. EDTA and methanol were purchased from Merck. Tris(2-carboxyethyl)phosphine hydrochloride, triethylammonium bicarbonate (TEABC), ammonium bicarbonate (ABC), PBS, sodium carbonate dihydrate (MgCl2), DL-DTT, HEPES, methyl methanethiosulfonate (MMTS), TFA, and HPLC grade ACN were purchased from Sigma-Aldrich. Formic acid (FA) and potassium dihydrogen phosphate (KH2PO4) were purchased from Riedel de Haen (Seelze, Germany). Water was obtained from a Milli-Q® Ultrapure Water Purification System (Millipore, Billerica, MA).

Animals and Preparation of Kidney Membrane Fraction—The PKD1loxP/loxP mice were generated according to Jiang et al. (30). Briefly a floxP site and a floxP-flanked mcl-1-neo cassette were introduced into introns 30 and 34, respectively, of the PKD1 locus to generate a conditional targeted mutation. Homozygotes were generated by a cross between heterozygotes and heterozygotes, and homzygotes were identified by PCR of tail genomic DNA. Mice were sacrificed by CO2 inhalation to obtain kidney samples. Renal tissue (~0.5 × 0.5 cm in size) was suspended in 5 ml of 10 mM homogenization buffer at pH 7.4 (0.25 M sucrose and 0.1 M EDTA) and then homogenized at 4 °C in a Tissuemizer homogenizer (BioSpec Products, Bartlesville, OK). Renal extract was centrifuged at 1,000 × g for 30 min at 4 °C, and then the supernatant was centrifuged at 100,000 × g for an additional 60 min at 4 °C in a Beckman L5-65 ultracentrifuge. The resulting crude membrane fraction pellet was resuspended in 50 μl of 6 M urea, 5 mM EDTA, and 2% (v/v) SDS in 0.1 M TEABC for gel-assisted digestion.

Isolation of HeLa Cell Membrane Proteins—HeLa cells were first washed three times with 10 ml of PBS and then scraped into hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, and 1 × protease inhibitor mixture (Calbiochem)) and homogenized with 50 strokes of a Dounce homogenizer. The membrane fraction was purified by two-step centrifugation. First nuclei were pelleted by centrifugation at 3,000 × g for 10 min at 4 °C. The postnuclear supernatant was mixed with 1.8 M sucrose to a final concentration of 0.25 M sucrose and was centrifuged for 1 h at 13,000 × g at 4 °C to pellet remaining membranes. The pellet was washed twice with 1 ml of ice-cold 0.1 M Na2CO3 (pH 11.5), dissolved in 50 μl of 90% (v/v) FA prior to the Bradford assay to determine the membrane protein concentration, and then vacuum-dried to obtain a membrane pellet for subsequent proteolysis reactions.

**Digestion of Membrane Proteins**—Four digestion methods were compared in this study. For Tube-Gel digestion (24), the membrane protein pellet was first dissolved in 50 μl of 25 mM ABC and 2% (w/v) SDS. The protein solution was mixed with 17.5 μl of acrylamide/bisacrylamide (40%, v/v, 29:1) solution, 2.5 μl of 1% (v/v) APS, and 1.07 μl of 100% TEMED in the tube without electrophoresis. The gel slice was cut into small pieces and washed several times with 25 mM ABC containing 50% (v/v) ACN. After drying in a SpeedVac, proteins were chemically reduced by the addition of 200 μl of 10 mM DTT at 60 °C for 30 min and alkylated by 200 μl of 55 mM iodoacetamide at room temperature in the dark for 30 min. The gel samples were further dehydrated with 100% ACN and then dried by SpeedVac. Proteolytic digestion was then performed with trypsin (protein:trypsin = 10:1, g/g) in 25 mM ABC with incubation overnight at 37 °C. Peptides were extracted from the gel using sequential extraction with 50 μl of 25 mM ABC, 100 μl of 0.1% (v/v) TFA in water, 150 μl of 0.1% (v/v) TFA in ACN, and 50 μl of 100% ACN, and the solutions were combined and concentrated by SpeedVac.

For gel-assisted digestion, the membrane protein pellet was resuspended in 50 μl of 6 M urea, 5 mM EDTA, and 2% (w/v) SDS in 0.1 M TEABC and incubated at 37 °C for 30 min for complete dissolution. Proteins were chemically reduced by adding 1.28 μl of 200 mM tris(2-carboxyethyl)phosphine and alkylated by adding 0.52 μl of 200 mM MMTS at room temperature for 30 min. To incorporate proteins into a gel directly in the Eppendorf vial, 18.5 μl of acrylamide/bisacrylamide solution (40%, v/v, 29:1), 2.5 μl of 10% (w/v) APS, and 1 μl of 100% TEMED was then applied to the membrane protein solution. The gel was cut into small pieces and washed several times with 1 ml of TEABC containing 50% (v/v) ACN. The gel samples were further dehydrated with 100% ACN and then completely dried by SpeedVac. Proteolytic digestion was then performed with trypsin (protein:trypsin = 10:1, g/g) in 25 mM TEABC with incubation overnight at 37 °C. Peptides were extracted from the gel using sequential extraction with 200 μl of 25 mM TEABC, 200 μl of 0.1% (v/v) TFA in water, 200 μl of 0.1% (v/v) TFA in ACN, and 200 μl of 100% ACN. The solutions were combined and concentrated by SpeedVac.

For methanol-assisted in-solution digestion (2), the membrane protein pellet was resuspended in 125 mM ABC. The suspension was first thermally denatured at 90 °C for 20 min, cooled on ice, and diluted by adding 100% methanol to produce a sample solution containing 60% (v/v) methanol. The final concentration of protein solution was 0.5 μg/μl. Tryptic digestion was performed at 37 °C for 5 h (protein:trypsin = 30:1, g/g). The suspension was saved, and the remaining pellet was subjected to digestion with trypsin (protein:trypsin = 50:1, g/g) for another 5 h. The supernatants of these two digestions were combined and concentrated by SpeedVac.

For SDS-assisted in-solution digestion (31), the membrane protein pellet was resuspended in 50 μl of 6 M urea, 5 mM EDTA, and 0.1% (w/v) SDS in 0.1 M TEABC and incubated at 37 °C for 30 min for complete dissolution. The membrane proteins were reduced and alkylated as described for the gel-assisted digestion. For subsequent tryptic digestion, the sample was diluted 6-fold with 0.1 M TEABC to reduce the concentration of urea and SDS. Proteolysis was performed with trypsin (protein:trypsin = 10:1, g/g) with incubation overnight at 37 °C. Urea and SDS were removed by strong cation exchange (SCX) chromatography and the Oasis Elution plate (Waters Corp.).

**iTRAQ Labeling and Fractionation by SCX Chromatography**—To label peptides with the iTRAQ reagent (Applied Biosystems, Foster City, CA), peptides labeled with iTRAQ 113, 114, 115, and 116 were loaded onto a SCX column (500 μl, 10 μm, 4 cm) equilibrated with 0.1% (v/v) TFA in water, 200 μl of 0.1% (v/v) TFA in 200 mM ACN, and the solutions were combined and concentrated by SpeedVac.
For protein fractionation, iTRAQ-labeled peptides were loaded onto a 2.1 × 200-mm polysulfonethyl A column containing 5-μm particles with 200-μm pore size (PolyLC, Columbia, MD). The peptides were eluted at a flow rate of 200 μl/min with a gradient of 0–25% buffer B (5 mM KH₂PO₄, 350 mM KCl, and 25% (v/v) ACN, pH 3.0) for 50 min followed by a gradient of 25–100% buffer B for 20 min. The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. Each fraction was vacuum-dried and then resuspended in 0.1% (v/v) TFA (40 μl) for further desalting and concentration using ZipTips™ (Millipore, Bedford, CA).

LC-ESI-MS/MS Analysis—iTRAQ-labeled samples were reconstituted in 6 μl of eluent buffer A (0.1% (v/v) FA in H₂O) and analyzed by μLC-MS/MS. Samples were injected into a 20-mm × 100-μm capillary trap column (Magic C₁₈, Michrom BioResources, Inc., Auburn, CA), separated on a 100-mm × 75-μm capillary column (Magic C₁₈, Michrom BioResources), and eluted with a linear gradient of 12–32% B (0.1% (v/v) FA in ACN) for 50 min at ~200–300 nl/min. An HP1100 solvent delivery system (Hewlett-Packard) was used with postcolumn flow splitting interfaced to a QSTAR Pulsar i mass spectrometer (Applied Biosystems). Peptide fragmentation by collision-induced dissociation was performed automatically using the information-dependent acquisition in Analyst QS v1.1 (Applied Biosystems). The method applied a 1-s TOF MS scan and automatically switched to three 1.5-s product ion scans (MS/MS) when a target ion reached an intensity of greater than 20 counts. TOF MS scanning was undertaken over the range 400–1600 m/z using a Q1 transmission window of 380 amu (100%). Product ion scans were undertaken over the range 110–1600 m/z at low resolution utilizing Q2 transmission windows of 90 amu (25%), 165 amu (25%), 330 amu (25%), and 660 amu (25%).

**Data Processing and Analysis**—For protein identification, data files from LC-ESI-MS/MS were batch-searched against the non-redundant International Protein Index (IPI) human sequence database v3.11 (32) (55,577 sequences) and mouse sequence database v3.15.1 (68,222 sequences) from the European Bioinformatics Institute using the MASCOT algorithm (v2.1.0, Matrix Science, London, UK). The peak list in MS/MS spectra generated under ESI-Q-TOF was extracted from AnalystQ3.1.1 (Applied Biosystems) with the default search parameters and validation criteria. All the false discovery rates were calculated using the Ingenuity Pathway Analysis Knowledge Base and databases including the Gene Ontology consortium and IPI database.

**RESULTS AND DISCUSSION**

**Selection and Optimization of Digestion Methods**

iTRAQ uses isobaric tagging chemistry at the peptide level; thus, effective solubilization, denaturation, and digestion are critical for high peptide yields. Therefore, the efficiency of various solubilization/digestion methods was first evaluated for the hydrophobic proteins that comprise the membrane proteome. We compared our gel-assisted digestion protocol with three recently published digestion methods, including SDS-assisted in-solution digestion (31), methanol-assisted in-solution digestion (2), and Tube-Gel digestion (24), all of which have shown enhanced solubilization and efficient digestion of membrane proteins. For SDS-assisted in-solution digestion (31), the membrane sample was dissolved in 0.1% SDS and 6 M urea followed by reduction and alkylation. To maintain trypsin activity, the membrane sample for SDS-assisted digestion
was diluted with TEABC to diminish the urea and SDS concentrations. SDS was removed by SCX chromatography to avoid interference during LC-MS/MS separation and identification. For methanol-assisted in-solution digestion (2), the membrane sample was first thermally denatured and then dissolved in 60% methanol to perform a two-step tryptic digestion. The remaining methanol was evaporated under vacuum before LC-MS/MS. For Tube-Gel digestion (24), the membrane sample was dissolved in 2% SDS and polymerized with acrylamide/polyacrylamide, APS, and TEMED directly in the tube without electrophoresis followed by standard in-gel digestion.

Our gel-assisted digestion method was adapted from the Tube-Gel digestion protocol (24). Compared with the original protocol, additional reduction/alkylation reactions were performed prior to polyacrylamide gel formation to 1) yield more complete protein denaturation and thus improve enzymatic digestion efficiency and 2) remove the detergent and reduction/alkylation reagents by subsequent in-gel washing steps. Importantly ammonium bicarbonate, the most commonly used buffer for protein digestion, was replaced with triethylammonium bicarbonate to avoid the reaction between the ammonium ion and iTRAQ reagents. To compare yields of digests, the tryptic peptides from the four methods were analyzed by single LC-MS/MS. Our study aims to develop an accurate quantitative proteomics platform; error-free protein identification is an essential prerequisite to confidently determine meaningful changes in a large scale data set. Thus, stringent protein identification criterion was chosen in the MASCOT database search such that all the false discovery rates achieved in the decoy database search were essentially 0% in this study.

Under the stringent thresholds in the protein identification, the comparison of results from three replicate experiments showed that gel-assisted digestion distinguished significantly more proteins than the Tube-Gel digestion, SDS-assisted in-solution digestion, and methanol-assisted in-solution digestion methods, identifying 114 ± 2, 50 ± 9, 42 ± 12, and 25 ± 7 non-redundant proteins, respectively (Fig. 1 and detailed information in supplemental Table 1). Among those proteins with known subcellular localizations by Gene Ontology annotations, a greater number of membrane proteins was identified by gel-assisted digestion (34 plasma membrane and 41 organelle membrane proteins) compared with Tube-Gel digestion (11 plasma membrane and 23 organelle membrane proteins), SDS-assisted in-solution digestion (15 plasma membrane and 11 organelle membrane proteins), and methanol-assisted in-solution digestion (12 plasma membrane and seven organelle membrane proteins). These comparative data clearly demonstrated that the peptide yield for membrane proteins was as follows: gel-assisted digestion > Tube-Gel digestion = SDS-assisted in-solution digestion > methanol-assisted in-solution digestion. Importantly the gel-assisted digestion recovered almost all membrane proteins (>90%) that were observed by the other three methods. Thus, the enhanced digestion efficiency of membrane proteins clearly demonstrated that gel-assisted digestion had the best performance among the four methods.

**Assessment of Reproducibility and Compatibility with iTRAQ Labeling by Quantitative Analysis of Four Replicate Preparations**

Variations in protein isolation, enzymatic digestion, and isotopic labeling can influence quantitation accuracy and reproducibility. Thus, we first assessed the reproducibility of the gel-assisted digestion technique, compatibility with iTRAQ labeling, and most importantly the precision of quantification. Four replicate sets of membrane proteins were independently purified from HeLa cells, separately subjected to gel-assisted digestion, labeled with one of the four isobaric reagents (iTRAQ114, iTRAQ115, iTRAQ116, or iTRAQ117), then pooled, and analyzed by LC-MS/MS. To
derive peptide and protein quantitative information, we used our recently developed software Multi-Q (33) to automatically process iTRAQ signature peaks, including peak detection, background subtraction, isotope correction, and peptide normalization, to remove systematic errors. These features allow for rapid interpretation of output from large proteomics data sets with minimal manual validation. Stringent quantification criteria were followed to determine iTRAQ ratios: 1) a MASCOT protein score of 68, 2) unique, labeled peptides, and 3) reporter ion intensity filter of at least 30 counts. It is noted that an essentially 0% false positive identification rate was achieved at the protein level based on the stringent identification criteria.

In the representative MS/MS spectrum of IGFGSFVEK (m/z 636.4; score, 82) from integrin β1 in Fig. 2a, the cluster of peaks in the mass window m/z 113–118 displayed the signature iTRAQ fragments in the ratio of 1.00:0.96:0.97:1.01. All the other quantified proteins also showed expected unitary ratios (supplemental Table 2). To determine the quantitation accuracy, the log ratio distribution for each quantified protein obtained from the weighted and normalized peptide ratio was plotted in Fig. 2b. All three pairwise ratios, iTRAQ115/iTRAQ114, iTRAQ116/iTRAQ114, and iTRAQ117/iTRAQ114, showed normal distributions with narrow variability in relative standard deviation (R.S.D.; p = 0.05) of 10, 11, and 13%, respectively. It is noted that the R.S.D. was even smaller than the typically reported 20% in iTRAQ experiments (15, 34, 35). The similarity of these distribution curves demonstrated the high reproducibility and precision of our quantitation strategy on the four independently prepared membrane samples. Based on a three-standard deviation model (p = 0.01), we considered a difference in abundance of 1.5-fold to indicate a statistically significant degree of up-regulation or down-regulation.

Large Scale Quantitative Membrane Proteomics by Gel-assisted Digestion, iTRAQ Labeling, and SCX Fractionation with Comparison with SDS-assisted In-solution Digestion

Work Flow and Experimental Design—To further establish a comprehensive method for large scale multiplexed quantitation of the membrane proteome, fractionation using a multi-dimensional peptide separation technique was incorporated into the optimized strategy described above. For performance evaluation as well as in-depth comparison between the newly developed gel-assisted digestion and other digestion methods, a purified membrane fraction from HeLa cells was divided into four equivalent lots. Two lots were digested using the gel-assisted digestion and subsequently labeled with iTRAQ114 and iTRAQ115. The remaining two lots were digested by SDS-assisted in-solution digestion and labeled with iTRAQ116 and iTRAQ117. We note that SDS-assisted in-solution digestion was chosen for comparison because it is the most widely adapted method for large scale isotopic labeling-based quantitation (2, 3, 23). Differences in iTRAQ ratios should reflect differences in peptide yield and quantitation accuracy between the two digestion methods. The results are described below.

Comprehensive Identification and Quantification of Membrane Proteins—A total of 696 non-redundant proteins (false discovery rate = 0%) were identified by combining LC-MS/MS data files from 42 SCX fractions. By quantitative analysis of 11,791 iTRAQ spectra using stringent criteria, Multi-Q determined iTRAQ ratios for 618 proteins (from 5,115 unique peptides). Further detailed information on peptide identification and quantification is given in supplemental Table 3. We used the TMHMM algorithm to predict the number of transmembrane helices for each identified protein. The analysis yielded 330 proteins that were predicted as integral mem-
brane proteins with at least one transmembrane helix (TMH), and a substantial number of proteins (112) had more than four (and up to 19) predicted TMHs. Further analysis with the Ingenuity Pathway Analysis Knowledge Base recognized the subcellular localization of 288 proteins; 190 of these proteins without a TMH were annotated as membrane proteins, including lipid-anchored and membrane-associated proteins. Taken together, our results quantified as many as 520 membrane proteins (i.e. 91%, exclusive of 8% unknown proteins) from the crude membrane fraction preparation.

To our knowledge, no other studies have achieved a quantitative proteomics analysis with such a high percentage of membrane proteins. Quantification of membrane proteins using the iTRAQ strategy has been reported recently (36). However, only 26–33% of the 137 ($p < 0.05$) and 149 ($p > 0.05$) quantified proteins in umbilical cord blood stem cells in that study were designated as membrane proteins. Although the authors attributed the low percentage of observed membrane proteins and the presence of other housekeeping proteins to cross-contamination from copurification of organelles during ultracentrifugation, we considered that the low yield of membrane proteins may also be due to deficiencies inherent in conventional in-solution digestion methods. This speculation can be inferred from our comparison of four digestion methods: the SDS-assisted in-solution digestion has very low peptide yield for membrane proteins, confirming the critical role of efficient digestion.

Enhanced Quantification of Transmembrane Domains—A crucial, yet largely unaddressed question in previous literature is whether a simple, robust, and flexible method can facilitate protease access to hydrophobic domains, such as the membrane-spanning peptides in multipass integral membrane proteins. Surprisingly we observed many examples of dramatically different yields for peptides belonging to the same protein (supplemental Table 3). The observations raised an interesting question of whether the improved yields from gel-assisted digestion varied in a structure-dependent manner. To address this issue, we constructed topological models of quantified peptides and correlated the extracellular/intracellular domain and TMH with iTRAQ ratios. The increases in yield of the majority of peptides in our gel-assisted protocol correlated strongly with TMH domains. Fig. 3 shows three examples of multpass integral membrane proteins. The monocarboxylate transporter 4 is a 12-pass integral membrane protein that maintains lactic acid flux during glycolysis (37) where failure to export the lactic acid leads to its accumulation in cells. Among the eight quantified peptides of the monocarboxylate transporter 4 (Fig. 3a), the greatest ratio between gel-assisted and SDS-assisted in-solution digestion occurred with the membrane-spanning peptides 87LLDLSVFR 114/117 ratio = 16.03; iTRAQ 115/iTRAQ117 ratio = 14.10).

Peptides close to TMH were also recovered at higher levels using our method (6AVSVFFK 114/iTRAQ117 ratio = 6.46; iTRAQ 115/iTRAQ117 ratio = 6.12), whereas the recoveries of the other peptides were 1–3-fold higher. Similarly significantly higher levels of more than 20-fold recoveries were achieved preferentially with the TMH-containing peptides 201ALNVLPFPYFR 211 (iTRAQ 114/iTRAQ117 = 22.41; iTRAQ 115/iTRAQ117 = 19.82) and 290LNPQFVEVLFR 300 (iTRAQ 114/iTRAQ117 = 29.80; iTRAQ 115/iTRAQ117 = 24.6) in isoform 4 of mitochondrial ATP-binding cassette (Fig. 3b). These profound effects were also observed in oligosaccharyltransferase STT3 (Fig. 3c). This strong correlation between the most dramatic changes in yield and the TMH peptides implies that the efficiency of cleavage of extremely hydrophobic peptides in proximity to the TMH was relatively enhanced by gel-assisted digestion.
As shown in Fig. 4a, SDS-assisted in-solution digestion (iTRAQ116 and iTRAQ117) yielded more low signal peptides (<30 counts, unquantified peptides), whereas gel-assisted digestion (iTRAQ114 and iTRAQ115) yielded greater numbers of quantified peptides with higher intensity. The difference is more dramatic for peptides having an ion count >300. Most of the 618 quantified proteins with high identification confidence were obtained at predominantly higher yield (ratio (R) > 1.5; R = iTRAQ114/iTRAQ117 or iTRAQ115/iTRAQ117) using our method. Supplemental Table 4 lists proteins that showed large differences in recovery between the two methods where 113 proteins had R ≥ 3.0, and only two proteins had R < 0.3. Many of the proteins detected in higher amounts by the gel-assisted method were membrane proteins involved in diverse molecular functions, e.g. receptors, enzymes, ion transporters, and signal transducers. Among these proteins, transporters (24 proteins) and receptors (17 proteins) were the predominant groups. Many proteins do not have annotated known functions, which highlights the lack of information on the membrane proteome and the need for advanced methodology to eliminate this deficiency.

To address whether detection of specific categories of proteins could be preferentially improved, the ratio distribution of quantifiable proteins in various subcellular localizations was plotted (Fig. 4b). The higher yield proteins (ratio, >1.5) were mainly membrane proteins (86.1%; exclusive of unknown and unclassified proteins). This observation may be attributable to more efficient dissolution and denaturation of the membrane proteome in the presence of high concentrations of SDS and urea, resulting in greater proteolysis. We also predicted the number of TMHs for these higher yield proteins to examine their hydrophobic characteristics (Fig. 4c). Using our method, the yield of multipass integral membrane proteins was greater than for proteins lacking a TMH. For example, membrane proteins with two or more TMHs comprised as much as 43.4% of the proteins with 3-fold higher yield (n = 77) and 28.6% of the proteins with 1.5–3-fold higher yield (n = 113), whereas no lower yield proteins contained two or more TMHs. Taken together, both trends reveal that gel-assisted digestion results in greater peptide yield for a greater number of hydrophobic membrane proteins, thereby facilitating subsequent peptide-level labeling with iTRAQ and providing more complete coverage of the membrane proteome.

**Improved Quantification of Membrane Proteins** — We further analyzed whether quantitation at the peptide level had a distinct advantage to provide better quantitation precision than
Quantitative Analysis of Membrane Proteins from Kidney Tissues of the ADPKD Mouse

Finally, we applied our method to a comparative membrane proteomics study of kidney tissues from the ADPKD mouse model. Using a gene-targeting strategy, we generated PKD1 mutant mice (PKD1L3/L3) with a polycystic kidney phenotype resembling human ADPKD (30). The PKD1L3/L3 mice appeared normal at birth but developed polycystic kidneys and did not survive past 4 weeks of age (data not shown). The mechanism of cyst formation has not been fully elucidated; however, altered membrane proteins have been hypothesized as one of the possible contributing factors (29, 39). To characterize differentially expressed membrane proteins that are involved in pathological changes in the ADPKD mouse model, we compared the profiles of plasma membrane fractions isolated from kidney at 14 days of age of two wild-type (Wt-1: iTRAQ114, Wt-2: iTRAQ116) and two PKD1L3/L3 mice (2L3-1: iTRAQ116, 2L3-2: iTRAQ117). Duplicate wild-type and PKD1L3/L3 mice were used to evaluate potential variations due to differences between individual mice, tissue sample preparations, and analytical procedures.

Using 400 μg of membrane proteins, 845 proteins were identified from 42 SCX fractions with a false discovery rate of 0% (see detailed information in supplemental Table 5). Only 11 proteins were identified with a single MS/MS spectrum (supplemental Fig. 3). The expression of the majority of 791 quantified proteins remained unchanged in PKD1L3/L3 mice, but 69 proteins were up-regulated by >2-fold, and 37 proteins were down-regulated by <2-fold. Membrane proteins constitute up to two-thirds of known drug targets, highlighting their critical pharmaceutical importance (40). Among the membrane protein drug targets, ion channels and receptors are the targets for 50% of currently marketed drugs (40). As shown in Table I, we observed significant up- or down-regulation of many ion channels and receptors in PKD1L3/L3 mice. One of these differentially expressed proteins, the Na+/K+-ATPase subunits, was confirmed by Western blotting. As shown in the time-dependent profile in Fig. 6, the levels of the Na+/K+-ATPase subunits were 2.2- and 1.8-fold lower, respectively, in the mutant compared with wild-type mice, whereas the β2 subunit expression was up-regulated in the mutant. These changes in expression levels of the Na+/K+-ATPase subunits were confirmed by Western blotting. As shown in the time-dependent profile in Fig. 6, the levels of the Na+/K+-ATPase α1, β1, and β2 subunits changed dramatically with disease progression.
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**Table I**

Partial summary of differentially expressed proteins from large scale analysis of the membrane fractions of wild-type mice versus PKD1 knock-out mice, a model for human polycystic kidney disease.

| Protein name                                      | Accession number | Protein score | Quantified peptides | Wt-2/Wt-1* | 2L3-1/Wt-1* | 2L3-2/Wt-1* | Subcellular localization |
|---------------------------------------------------|------------------|---------------|---------------------|------------|------------|------------|-------------------------|
| Sodium/potassium-transporting ATPase α1 chain     | IPI00311682      | 5,067         | 108                 | 1.14 ± 0.19 | 0.65 ± 0.16 | 0.61 ± 0.16 | Plasma membrane          |
| Sodium/potassium-transporting ATPase γ chain      | IPI00227916      | 102           | 6                   | 1.12 ± 0.22 | 0.54 ± 0.12 | 0.50 ± 0.10 | Plasma membrane          |
| Receptor                                          |                  |               |                     |            |            |            |                         |
| Fibrinogen β chain precursor                      | IPI00279079      | 315           | 6                   | 1.17 ± 0.27 | 12.95 ± 6.53 | 3.28 ± 1.41 | Extracellular space      |
| Fibrinogen, α polypeptide                         | IPI00115522      | 237           | 10                  | 1.39 ± 0.27 | 9.14 ± 4.23 | 2.71 ± 0.87 | Extracellular space      |
| Fibrinogen γ chain precursor                      | IPI00122312      | 253           | 19                  | 1.14 ± 0.14 | 7.61 ± 3.53 | 2.33 ± 0.79 | Extracellular space      |
| Macrophage mannose receptor 1 precursor           | IPI00126186      | 74            | 4                   | 1.19 ± 0.22 | 4.75 ± 3.53 | 4.21 ± 2.85 | Plasma membrane          |
| Epidermal growth factor receptor precursor        | IPI00121190      | 73            | 3                   | 1.11 ± 0.15 | 3.04 ± 0.28 | 2.19 ± 0.17 | Plasma membrane          |
| Lysosome membrane protein 2                       | IPI00127447      | 88            | 6                   | 1.10 ± 0.18 | 2.52 ± 0.90 | 2.50 ± 0.98 | Plasma membrane          |
| Integrin β1 precursor                             | IPI00132474      | 326           | 9                   | 1.25 ± 0.17 | 2.30 ± 0.36 | 2.02 ± 0.35 | Plasma membrane          |
| Neuregulin 1                                      | IPI00131459      | 177           | 5                   | 1.13 ± 0.17 | 0.61 ± 0.36 | 0.51 ± 0.03 | Plasma membrane          |
| Transporter                                       |                  |               |                     |            |            |            |                         |
| Sideroflexin-3                                    | IPI00126115      | 99            | 2                   | 1.29 ± 0.11 | 2.73 ± 0.90 | 2.52 ± 0.98 | Plasma membrane          |
| Ammonium transporter Rh type C                     | IPI00311731      | 68            | 2                   | 0.96 ± N/A  | 2.25 ± 0.60 | 1.40 ± 0.48 | Plasma membrane          |
| Sodium/glucose cotransporter 2                    | IPI00124340      | 182           | 8                   | 1.46 ± 0.19 | 0.68 ± 0.09 | 0.75 ± 0.06 | Plasma membrane          |
| Sodium-dependent neutral amino acid transporter B | IPI00108676      | 76            | 8                   | 1.30 ± 0.11 | 0.62 ± 0.06 | 0.69 ± 0.05 | Plasma membrane          |
| Electrogenic sodium bicarbonate cotransporter 1   | IPI00314749      | 823           | 31                  | 1.23 ± 0.39 | 0.60 ± 0.16 | 0.71 ± 0.18 | Plasma membrane          |
| Solute carrier family 2, facilitated glucose      | IPI00311809      | 170           | 10                  | 1.23 ± 0.13 | 0.58 ± 0.08 | 0.58 ± 0.10 | Plasma membrane          |
| transporter member 2                              | IPI00227082      | 74            | 6                   | 1.22 ± 0.15 | 0.54 ± 0.03 | 0.80 ± 0.07 | Plasma membrane          |
| Solute carrier family 5 (iodide transporter),     | IPI00119459      | 78            | 6                   | 1.48 ± 0.22 | 0.52 ± 0.08 | 0.55 ± 0.05 | Plasma membrane          |
| member 8                                          | IPI00323646      | 70            | 6                   | 1.39 ± 0.17 | 0.50 ± 0.11 | 0.69 ± 0.11 | Plasma membrane          |
| Solute carrier family 12 member 1                 | IPI00173140      | 206           | 14                  | 1.26 ± 0.18 | 0.49 ± 0.10 | 0.58 ± 0.10 | Plasma membrane          |
| Solute carrier family 22 (organic ion/cation       | IPI00117817      | 233           | 14                  | 1.48 ± 0.31 | 0.49 ± 0.18 | 0.68 ± 0.16 | Plasma membrane          |
| transporter), member 12                          |                  |               |                     |            |            |            |                         |
| Organic cation transporter-like protein 2         | IPI00310201      | 84            | 8                   | 1.23 ± 0.16 | 0.46 ± 0.09 | 0.67 ± 0.06 | Plasma membrane          |
| Dimethylamine monooxygenase (N-oxide-forming) 1   | IPI00116432      | 295           | 11                  | 1.28 ± 0.21 | 0.32 ± 0.06 | 0.59 ± 0.10 | Organelle membrane       |
| Sodium-dependent phosphate transport protein 2A   | IPI00121337      | 74            | 4                   | 1.45 ± 0.35 | 0.30 ± 0.10 | 0.55 ± 0.20 | Plasma membrane          |
| Solute carrier family 13 member 3                 | IPI00129564      | 71            | 3                   | 0.67 ± 0.43 | 0.26 ± 0.45 | 0.28 ± 0.49 | Plasma membrane          |

* a ITRAQ ratio. Wt-1 and Wt-2 indicate duplicate wild-type mice, and 2L3-1 and 2L3-2 indicate duplicate PKD1 mutant mice.

b The subcellular location of differentially expressed proteins was categorized by Gene Ontology.

c Not available.

To further explore the possibility that our method could facilitate the identification of drug targets and lead to new and improved pharmaceutical treatments, these membrane proteins were analyzed by the Ingenuity Pathway Analysis Knowledge Base for correlation with diseases and drugs. As shown in Table II, 23 proteins were known targets of various drugs in clinical use or in preclinical trials with animal models associated with various diseases. Among the 23 proteins, the levels of 11 (47.8%) differed between normal and ADPKD mice. Of particular interest were several proteins that have been demonstrated to attenuate renal enlargement and renal dysfunction (43) or reverse cyst formation in mouse models (27); these proteins were epidermal growth factor receptor (EGFR; 2L3-1/Wt-1 = 3.04, 2L3-2/Wt-1 = 2.19), cyclooxygenase (COX: 2L3-1/Wt-1 = 2.49, 2L3-2/Wt-1 = 2.77), and Na⁺/K⁺-ATPase (ATPα1: 2L3-1/Wt-1 = 0.65, 2L3-2/Wt-1 = 0.51; ATPβ1: 2L3-1/Wt-1 = 0.54, 2L3-2/Wt-1 = 0.50).

**Fig. 6.** Verification of expression levels of Na⁺/K⁺-ATPase α1, β1, and β2 in the kidney of wild-type versus PKD1L3/L3 mice. Membrane fractions were extracted from each wild-type and PKD1L3/L3 mouse at the indicated time and analyzed by Western blotting.

The levels of subunits α1 and β1 in PKD1L3/L3 mice were almost nil by postnatal day 21, whereas that of β2 increased substantially between postnatal days 14 and 25.
| Gene name | Description | 2L3-1/Wt-1 a | 2L3-2/Wt-1 b | Drugs | Diseases a |
|-----------|-------------|-------------|-------------|--------|------------|
| FGB       | Fibrinogen β chain | 12.95       | 3.28        | Thrombin | Congenital afibrinogenemia, hypofibrinogenemia |
| FGG       | Fibrinogen γ chain | 7.61        | 2.33        | Thrombin | Congenital afibrinogenemia, hypofibrinogenemia, hemophilia, dysfibrinogenemia, liver cancer, hereditary renal amyloidoses |
| FGA       | Fibrinogen α chain | 9.14        | 2.71        | Thrombin | Congenital afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, liver cancer, hereditary renal amyloidoses |
| ADH1C     | Alcohol dehydrogenase 1C (class I), γ polypeptide | 3.53        | 2.57        | Fomepizole | Lung cancer |
| HSD3B2    | Hydroxy-Δ3-steroid dehydrogenase, 3β- and steroid Δ-5-isomerase 2 | 2.91        | N/A c | Trilostane | Cushing syndrome |
| EGFR      | Epidermal growth factor receptor | 3.04        | 2.19        | Cetuximab, AEE 788, panitumumab, BMS-599626, ARRY-334543, XL647, erlotinib, gefitinib, KI-272, PD 153035, lapatinib, vandetanib, erlotinib | ADPKD, lung cancer, head and neck cancer, breast cancer, ovarian cancer |
| SLC9A1    | Solute carrier family 9 (sodium/hydrogen exchanger), member 1 | 3.30        | N/A | Amiloride, amiloride/hydrochlorothiazide | Alkalization, heart failure, essential hypertension, epileptic seizure |
| COL6A1    | Collagen, type VI, α1 | 2.78        | 3.55        | Collagenase | Prostate cancer |
| TUBA1A    | Tubulin, α1 | 2.44        | 1.60        | Epothilone B, ixabepilone, colchicine/probenecid, XRP9881, ET389, AL 108, EC145, NPI-2358, mlataxel, TPI 267, TTI-237, docetaxel, vinflunine, vinorelbine, vincristine, vinblastine, paclitaxel, podophyllotoxin, colchicine | Prostate cancer, breast cancer, human Leukemia, lung cancer, colorectal cancer, renal cell cancer, Parkinson disease |
| PLG       | Plasminogen | 2.27        | 1.72        | Tissue plasminogen activator, tenecteplase, aprotinin, ε-aminocaproic acid, reteplase | Metastasis |
| COX       | Prostaglandin-endoperoxide synthase 1 (cyclooxygenase) | 2.49        | 2.77        | Acetaminophen/pentazocine, acetaminophen/celecoxib, asparin/butalbital/caffeine, acetaminophen/caffeine/dihydrocodeine, aspirin/hydrocodone, aspirin/oxycodeine, acetaminophen/aspirin/caffeine, aspirin/pravastatin, acetaminophen/dexibrompheniramine/pseudoephedrine, aspirin/meprobamate, aspirin/caffeine/progesterone, aspirin/butalbital/caffeine/codeine, aspirin/caffeine/dihydrocodeine, chlorpheniramine/ibuprofen/pseudoephedrine, licofelone, menenatrenone, diclofenac/misoprostol, acetaminophen/butalbital/caffeine, hydrocodone/ibuprofen, acetaminophen/hydrocodone, acetaminophen/trimadol, acetaminophen/codeine, acetaminophen/oxycodeine, acetaminophen/propranolol, nitroaspirin, ketoprofen, diclofenac, naproxen, meclofenamic acid, meloxicam, dipeyrone, acetaminophen, mefenamic acid, diflunisal, ibuprofen, indomethacin, sulfasalazine, piroxicam, aspirin, zomepirac, aspirin/methocarbamil, aspirin/caffeine/orphenadrine, aspirin/carisoprodol, aspirin/carisoprodol/codeine, acetaminophen/butalbital, balsalazide, aspirin/dipyridamole, acetaminophen/butalbital/caffeine/codeine, flurbiprofen, phenacetin, sulindac, nabumetone, etodolac, tolmetin, ketorolac, 5-aminosalicylic acid, fenoprofen, salicylic acid, verteporfin, acetaminophen/chlorpheniramine/hydrocodeine/phenylephrine/caffeine, bromfenac | Lung cancer, ovarian cancer, autosomal dominant polycystic kidney disease |

**NOTE:** The table may contain errors or omissions. Please double-check the source for the most accurate information.

**Molecular & Cellular Proteomics 7.10 1993**
An Efficient Quantitative Strategy for Membrane Proteomics

Systemic Manifestation of the Altered Membrane Proteome Provides Insight into the Molecular Mechanism and Opportunities for Effective Pathophysiology-based Therapy for ADPKD

To date, effective therapy for ADPKD is lacking with the exception of dialysis and kidney transplantation. The development of better therapeutic treatment is founded on an understanding of the molecular pathophysiological mechanism for the development of ADPKD. Although the mechanisms underlying ADPKD pathology are not well understood, a close examination of the differentially expressed membrane proteins quantified in the current comparative approach reveals potential correlations with proteins associated with the pathogenesis of ADPKD in previous literature. Fig. 7 summarizes some of these proteins that have been confirmed to be involved in the major abnormalities in epithelial cell proliferation and apoptosis, cell-cell and cell-matrix interactions, abnormal ion and fluid secretion, and alterations in membrane protein polarity (38).

A recent study has suggested that polycystin-1, an 11-TMH membrane receptor encoded by PKD1, acts as a renal epithelial cell membrane mechanoreceptor, sensing morphogenetic cues in the extracellular environment at the basal surface in focal adhesion complexes, at the lateral surface in cell adherens junctions, and in the lumen at the apical primary cilium (44). Normal function of PKD genes and proteins leads to the formation of normal kidneys and the down-regulation of many fetal genes. The disruption of polycystin multiprotein complexes and downstream signaling may lead to a failure to down-regulate the expression of fetal proteins, including Na⁺/K⁺-ATPase and EGFR. Increased proliferation is an important component of cystic expansion in ADPKD. Overexpression and mislocalization of EGFR complexes have been observed in cystic epithelia from human, mouse, and other animal models of ADPKD, and such abnormalities subsequently initiate signaling cascades with concomitant cell proliferation and cyst formation/enlargement (39, 45). The observed cyst enlargement and increased expression of EGFR in both ADPKD mutant mice we tested (2L3-1/Wt-1 = 3.00, 2L3-2/Wt-1 = 2.24; also see Table II) are in agreement with the above studies. In normal fluid secretion, the net reabsorption of fluid in renal cyst is brought about by sodium ion gradients established by the Na⁺/K⁺-ATPase in the basolateral tubular cell membrane. Malfunction of this pump may cause the main symptom of ADPKD: fluid accumulation in the renal cyst. The Na⁺/K⁺-ATPase consists of α1 and β1 subunits in normal adult and fetal kidneys, respectively. We found that the level of subunits α1 and β1 in the cystic kidney was drastically decreased compared with that in normal kidneys (see above). Down-regulation of subunit α1 in cystic epithelial cells may decrease the sodium gradient, further leading to fluid accumulation in renal cysts. However, expression of subunit β2 was increased in cystic kidneys (Fig. 6). Because the β2 subunit is highly expressed in normal fetal kidneys (30), this result suggests a degree of either undifferentiation or dedifferentiation in the renal cystic epithelium.

TABLE II—continued

| Gene name | Description | 2L3-1/Wt-1* | 2L3-2/Wt-1* | Drugs | Diseases
|-----------|-------------|-------------|-------------|------|---------|
| MAOA      | Monoamine oxidase A | 1.64 | 1.59 | Ladostigil, 1-ethylphenoxathiin 10,10-dioxide, dextroamphetamine, procainamide, triamcinolone, phenelzine, isocarboxazid, benzphetamine, N-(2-indanylglycinamide | Parkinson disease, attention deficit disorder |
| FOLH1     | Folate hydrolase (prostate-specific membrane antigen) 1 | 1.17 | 1.38 | Capromab pendetide | Oral cancer, head and neck cancer |
| HMOX2     | Heme oxygenase (decycling) 2 | 1.16 | 1.34 | Tin mesoporphyrin | Hyperbilirubinemia, Parkinson disease |
| HSP90B1   | Heat shock protein 90 kDa β (Grp94), member 1 | 0.97 | 1.22 | 17-Dimethylaminoethylamino-17-deoxyxeglandamycin, IP-504 | Multiple myeloma, rheumatoid arthritis, Hypertriglyceridemia, obesity |
| DGAT1     | Diacylglycerol O-acetyltransferase homolog 1 | 1.21 | 1.35 | Omacor | Ocular hypertension, renal tubular acidosis |
| CA2       | Carbonic anhydrase II | 0.94 | 0.82 | Methazolamide, hydrochlorothiazide, acetazolamide, chlorothiazide, dorzolamide, trimolol, brinzolamide, chlorthaldone, benzthiazide, sulfacetamide, thioramate | N/A, not available. |
| DPP4      | Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2) | 0.81 | 0.96 | Saxagliptin, talabostat, SYR-322, sitagliptin | Lung cancer, neoplasia |
| ATP1A1    | Na⁺/K⁺-ATPase α1 chain | 0.65 | 0.61 | Cardiotonic steroid | Cardiovascular disease, cancer |
| PPIA      | Peptidylprolyl isomerase A (cyclophilin A) | 0.59 | 0.83 | N-Methyl-4-Ile-cyclosporin | Allergy, hepatitis C |

* iTRAQ ratio. Wt-1 and Wt-2 indicate duplicate wild-type mice, and 2L3-1 and 2L3-2 indicate duplicate PKD1 mutant mice.

b The references are listed in supplemental Table 4.

c N/A, not available.
Alterations in epithelial cell adhesion and migration also are important characteristics in human and mouse PKD cysts (39). Cell-matrix interactions in focal adhesions are mediated by integrin receptors. Apparently the up-regulation of \( \beta^\text{1 integrin (ITGB; 2L3-1/Wt-1} \), \( \beta^\text{1 integrin (ITGB; 2L3-2/Wt-1} \) in both mutant mice is consistent with the higher level of \( \beta^\text{1 integrin observed by Wilson et al. (46) in ADPKD-derived cells. The up-regulation may induce characteristically increased \( \beta^\text{1 integrin-mediated adhesion to the extracellular matrix (47). In addition, we observed up-regulation of integrin-interacting proteins, including talin (TLN1; 2L3-1/Wt-1 = 1.47, 2L3-2/Wt-1 = 1.51), vinculin (VCL; 2L3-1/Wt-1 = 2.03, 2L3-2/Wt-1 = 1.91), and \( \alpha^\text{-actinin (ACTN1; 2L3-1/Wt-1 = 2.73, 2L3-2/Wt-1 = 1.96), which have been suggested to form a multiprotein complex upon binding to matrix components (48). Reduced cell-cell adhesion has been hypothesized as a characteristic of a dedifferentiated phenotype (39). In addition to association with EGFR in normal kidney cells, polycystin-1 is also essential to maintain a fully differentiated polarized renal epithelium by forming a complex with epithelial cell adhesion molecules including E-cadherin and its associated catenins through its cytoplasmic tail (49). In primary cells from ADPKD patients, Wandinger-Ness and co-workers (49) observed that the multiprotein complex was disrupted and that both polycystin-1 (PC1; 2L3-1/Wt-1 = 0.87, 2L3-2/Wt-1 = 0.99) and E-cadherin (CDH1; 2L3-1/Wt-1 = 1.38, 2L3-2/Wt-1 = 1.66) were depleted from the plasma membrane, characterized by subcellular sequestration to an intracellular compartment (50). In our study, the levels of polycystin-1 and E-cadherin were unchanged in the ADPKD kidney, which may echo previous observations that total cellular levels of these two proteins are similar in normal human kidney and ADPKD epithelia. Interestingly another cadherin member, the kidney-specific cadherin, was clearly down-regulated (2L3-1/Wt-1 = 0.53, 2L3-2/Wt-1 = 0.62). Thus, clarification of the roles of different cadherins in abnormal adherens junctions may have to wait until further ultracentrifugation experiments can specifically isolate the plasma membrane with subsequent analysis of expression levels in normal versus ADPKD mice.

Although the preliminary results presented here suggest that certain differentially expressed membrane proteins are associated with the pathogenesis of ADPKD, the identification of additional potential drug target candidates will speed the discovery of an effective therapy. Indeed recent advances in the molecular mechanism of ADPKD pathogenesis have led to new therapeutic opportunities for prevention or retardation of the disease (51). As shown in Table II, three membrane proteins that displayed altered expression levels have already been targeted in a potential treatment for ADPKD. The first example is the abnormally polarized hyperactive apical EGFR. The successful use of a specific EGFR inhibitor in reversing excessive cellular adhesion and sluggish migration defects

**Fig. 7.** Partial pathways of differentially expressed proteins that have been confirmed to be involved in the major abnormalities in epithelial cell proliferation and apoptosis, cell-cell and cell-matrix interactions, abnormal ion and fluid secretion, and alterations in membrane protein polarity in polycystic kidney disease. The ratios, (2L3-1/Wt-1)/(2L3-2/Wt-1), in the figure indicate the fold-change of protein expression level in PKD knock-out compared with control mice. ATP1, Na\(^+\)/K\(^-\)-ATPase; FAK, focal adhesion kinase; ITGB, integrin \( \beta^\text{1, ITGA, integrin \( \alpha^\text{.} \)
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has been reported in human ADPKD epithelia in vitro as well as reversals of cyst formation in mouse models of PKD (27). Furthermore the recent discovery of the role of EGFR as a signal transducer that modulates cell growth, cell adhesion, and apoptotic threshold opens new avenues for drug development (41). The expression of cyclooxygenases (COX-1 and COX-2), enzymes that regulate the formation of physiologically active prostaglandins, has been found to be up-regulated (COX-1) and down-regulated (COX-2) in rodent models of PKD. Inhibition of COX-2 by a steroidal anti-inflammatory drug, methylprednisolone, attenuated renal enlargement and renal dysfunction in two rodent models of renal cystic disease (43), thereby demonstrating its potential as a target for treatment in PKD. Although some of the differentially expressed proteins in ADPKD mice, such as Na+/K+-ATPase, have been demonstrated to play a critical role in the development of the cystic phenotype, they have not been tested as potential targets for treatment of ADPKD or renal disease. Because Na+/K+-ATPase is a clinically relevant drug target for cardiovascular disease (52) as well as cancer (53), whether it can potentially be targeted for ADPKD remains open to further investigation. Given the possibilities offered by our new technique for more efficient identification and quantification of large numbers of transporters/receptors, we expect to systematically decipher the mechanism of cystogenesis and identify additional drug target candidates in the near future.

Conclusion

In this study, we demonstrated a comprehensive and high throughput methodology for multiplexed quantitation of a membrane proteome that offers both high reproducibility and superior consistency. The protocol offers the advantages of 1) compatibility with high concentrations of detergent and salts for efficient solubilization, denaturation, and digestion of membrane proteins and 2) improved proteome coverage and quantitation accuracy by peptide-level isotopic tagging of amino groups. We are presently applying this strategy to several other quantitative membrane proteomics studies, including lipid rafts and tissues in several physiological states. Although bioinformatics analysis/validation with other biochemical methods is not yet complete, all of these studies have yielded large scale, reproducible, and robust quantitative analysis of extremely hydrophobic proteins. The new approach offers an improved quantitation platform to investigate differential expression of membrane proteins in cells, biofluids, and tissues under different environmental or pathophysiological conditions.

* This work was supported by Academia Sinica and the National Science Council in Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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