Proteomic analysis of brush-border membrane vesicles isolated from purified proximal convoluted tubules

Scott J. Walmsley,1 Corey Broeckling,2 Ann Hess,3 Jessica Prenni,2,4 and Norman P. Curthoys1,4

1Cell and Molecular Biology Program, 2Proteomics and Metabolomics Facility, 3Department of Statistics, and 4Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado

Submitted 14 December 2009; accepted in final form 6 March 2010

Walsmsley SJ, Broeckling C, Hess A, Prenni J, Curthoys NP. Proteomic analysis of brush-border membrane vesicles isolated from purified proximal convoluted tubules. Am J Physiol Renal Physiol 298: F1323–F1331, 2010. First published March 10, 2010; doi:10.1152/ajprenal.00711.2009.—The renal proximal convoluted tubule is the primary site of water, electrolyte and nutrient reabsorption and of active secretion of selected molecules. Proteins in the apical brush-border membrane facilitate these functions and initiate some of the cellular responses to altered renal physiology. The current study uses two-dimensional liquid chromatography/mass spectrometry to compare brush border membrane vesicles isolated from rat renal cortex (BBMVCTX) and from purified proximal convoluted tubules (BBMVPCT). Both proteomic data and Western blot analysis indicate that the BBMVCTX contain apical membrane proteins from cortical cells other than the proximal tubule. This heterogeneity was greatly reduced in the BBMVPCT. Proteomic analysis identified 193 proteins common to both samples, 21 proteins unique to BBMVCTX, and 57 proteins unique to BBMVPCT. Spectral counts were used to quantify relative differences in protein abundance. This analysis identified 42 and 50 proteins that are significantly enriched (p values ≤0.001) in the BBMVCTX and BBMVPCT, respectively. These data were validated by measurement of y-glutamyltranspeptidase activity and by Western blot analysis. The combined results establish that BBMVPCT are primarily derived from the proximal convoluted tubule (S1 and S2 segments), whereas BBMVCTX include proteins from the proximal straight tubule (S3 segment). Analysis of functional annotations indicated that BBMVPCT are enriched in mitochondrial proteins and enzymes involved in glucose and organic acid metabolism. Thus the current study reports a detailed proteomic analysis of the brush-border membrane of the rat renal proximal convoluted tubule and provides a database for future hypothesis-driven research.

THE PROXIMAL TUBULE IS THE most abundant segment of the nephron within the renal cortex (23). It is composed of polarized epithelial cells and consists of three subsegments (S1, S2, and S3) that differ in protein composition and function. The primary functions of the entire proximal tubule are the recovery of ~80% of the fluid and electrolytes from the glomerular filtrate, the reabsorption of >99% of the filtered glucose and other nutrients, and the active secretion of selected molecules. These processes are facilitated by a unique set of largely Na+-independent transporters in the basolateral membrane that promote exchange of solutes with blood and a separate set of primarily Na+-dependent transporters in the apical brush-border membrane that facilitate exchange with the glomerular filtrate. In addition, the brush-border membrane also contains multiple hydrolases and proteins involved in receptor-mediated signaling.

Previous analyses of the brush-border membrane have primarily utilized brush-border membrane vesicles (BBMV) that were isolated by MgCl2 precipitation from renal cortical homogenates (4). Such studies have included the functional characterization of transporters such as the Na+/H+ exchanger (6), and of various peptidases and disaccharidases (22). While these studies sought to characterize the function of a single protein, a recent analysis utilized a shotgun approach to define the proteome of BBMV isolated from rat renal cortex (12). This approach offers the potential of creating a comprehensive inventory of the protein composition of the brush-border membrane. In addition, a thorough proteomic characterization of highly purified brush-border membranes would provide additional insight into the processes mediated by the proximal tubule during normal physiology. It would also provide the basis for characterizing alterations in the proteome that are associated with the loss of specific functions during pathological conditions such as hypoxic or toxic injury to the proximal tubule.

However, due to the presence of multiple cell types and the subtle differences between the segments of the proximal tubule, it may be difficult to accurately define the protein composition using BBMV isolated from renal cortex. For example, BBMV prepared from microdissected proximal convoluted and proximal straight tubules exhibit differences in kinetic parameters of glucose uptake, indicative of cell-specific expression of different isoforms (13). In addition, immunofluorescence studies have demonstrated that numerous proteins are preferentially expressed in the individual segments of the proximal tubule. For example, y-glutamyltranspeptidase expression is greater in the proximal straight tubule (S3 segment) than in the proximal convoluted tubule (S1 and S2 segments) (9, 10, 35). Together, these results demonstrate that an accurate assessment of the localization of specific proteins by proteomic analysis may require the further enrichment of BBMV from specific segments of the proximal tubule.

In this study, two-dimensional liquid chromatography/mass spectrometry (LC-MS/MS) was used to compare the proteome of BBMV prepared from rat renal cortex (BBMVCTX) and from isolated proximal convoluted tubules (BBMVPCT). This approach, along with immunoblot analysis of marker proteins, indicated that the BBMVCTX preparation also contains apical membranes that are derived from cells of the renal cortex other than the proximal tubule. However, the initial isolation of proximal convoluted tubules by Percoll gradient centrifugation was sufficient to remove the identified contaminants from the
BBMV\textsubscript{PCT} preparation. The comparative proteomic analysis also revealed significant differences between the two samples. This analysis, along with the observed differences in γ-glutamyltranspeptidase activity, indicates that the BBMV\textsubscript{PCT} are derived primarily from the S1 and S2 segments of the proximal tubule. Conversely, only the BBMV\textsubscript{CTX} contain proteins that are expressed solely in the S3 segment and exhibit higher levels of proteins that are enriched in the proximal straight tubule. Finally, an analysis of functional annotations indicates that BBMV\textsubscript{PCT} are enriched in enzymes of glucose metabolism, organic acid catabolism, and mitochondrial proteins. Therefore this study provides the first detailed proteomic analysis of the brush-border membrane of the rat renal proximal convoluted tubule.

**MATERIALS AND METHODS**

*Materials.* Rabbit polyclonal antibodies vs. rBAT, basolateral glucose transporter (GLUT2), Na\(^+/\)H\(^+\) exchanger regulatory factor-1 (NHERF1), and endoplasmic reticulum marker calnexin (CNX; H-70) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Chemicon International (Billerica, MA), Millipore (Temecula, CA), and Abcam (Cambridge, MA), respectively. A mouse monoclonal antibody to the 70-kDa subunit of succinate dehydrogenase was from Mitosciences (Eugene, OR). Rabbit polyclonal antibodies raised against aquaporin-2, thiazide-sensitive Na\(^+/\)Cl\(^-\) cotransporter, and Na\(^+\)/K\(^+\)2Cl\(^-\) co-transporter-2 were kindly provided by Dr. Mark Knepper (National Institutes of Health, Bethesda, MD). Rabbit antibodies vs. rat γ-glutamyltranspeptidase were obtained from Dr. Rebecca Hughey (University of Pittsburgh). Mouse monoclonal antibodies to Na\(^+/\)H\(^+\) exchanger-3 (NHE3) were kindly provided by Dr. Orson Moe (Southwestern Medical Center, Dallas, TX). Male Sprague-Dawley rats (~200 g) were obtained from Charles River Laboratories (Kingston, NY) and were fed rodent chow (Harlan-Teklad, Madison, WI) and tap water. All procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University.

**Purification of proximal convoluted tubules.** Rat renal proximal convoluted tubules were isolated by Percoll density gradient centrifugation (11). Briefly, ~1-mm\(^3\) pieces of excised kidney cortex were incubated in PBS containing 5 mM glucose, 1 mg/ml bovine serum albumin, 0.1 mg/ml DNase, 2 mg/ml collagenase B (Roche Diagnostics, Mannheim, Germany), 1 mM heptanoic acid, 1 mM phenylmethysulfonyl fluoride, and 1 mM sodium orthovanadate. The resulting tubules were washed twice in PBS containing 5 mM glucose to remove collagenase and then resuspended in an osmotically and pH-balanced PBS solution containing 5 mM glucose, 45% Percoll (Sigma Life Sciences), and 10 mM HEPES, pH 7.4. After centrifugation, the tubules were recovered from a band that formed near the bottom of the gradient and were washed twice with PBS containing 5 mM glucose to remove the Percoll.

**Isolation of BBMV.** BBMV were prepared using the standard method of MgCl\(_2\) precipitation (4, 5). Excised kidney cortex or purified proximal convoluted tubules were resuspended in 10 volumes (vol/wet wt.) of a solution containing 300 mM mannitol, 5 mM EGTA, 1 mM phenylmethysulfonyl fluoride, 1 mM sodium orthovanadate and 12 mM HEPES, pH 7.1. After polytron homogenization (90 s, setting 5), the homogenate was diluted twofold with H\(_2\)O and then MgCl\(_2\) was added to yield a final concentration of 12 mM. The mixture was then incubated on ice for 15 min with intermittent and gentle mixing. Following centrifugation at 3,000 g for 10 min at 4°C to remove mitochondria and cellular debris, the resultant supernatant was centrifuged at 30,000 g for 40 min at 4°C to pellet the BBMV. The pellet was then resuspended in 1 volume of 150 mM mannitol, 2.5 mM EGTA, and 6 mM HEPES, pH 7.1 and homogenized with 15 passes of a glass teflon homogenizer. The BBMV were again precipitated by addition of 12 mM MgCl\(_2\) and repetition of the incubation and centrifugation steps. The final pellet was resuspended in the previous mannitol buffer, and the BBMV were stored at −80°C.

γ-Glutamyltranspeptidase assay and immunoblot analyses. Aliquots of cortical homogenate and the isolated BBMV were assayed for protein (30) and γ-glutamyltranspeptidase activity (29). The specific activity (μmol·min\(^{-1}·mg\(^{-1}\)) was determined by quantifying the liberation of p-nitroaniline from γ-glutamyl-p-nitroaniline (Sigma-Aldrich) with glycyglycine (Sigma-Aldrich) as the acceptor. For immunoblot analyses, samples containing 20 μg of protein were separated by 7% or 10% SDS-PAGE. Following transfer to polyvinylidene difluoride membranes (Immobilon-FL, Millipore), the blots were incubated overnight with either mouse monoclonal or rabbit polyclonal antibodies. Either 680 or 800 Dylight-conjugated goat anti-mouse or goat anti-rabbit IgG (Pierce) was used as a secondary antibody [1:10,000 (vol/vol)]. The resulting complexes were visualized and quantified using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE).

**Sample preparation and MS analysis.** Aliquots of BBMV containing 100 μg of protein were denatured by heating at 95°C for 5 min. After cooling to room temperature, the samples were dried and reconstituted to 3 mg/ml in 8 M urea. The ultrasonicated samples were reduced (14 mM dithiothreitol, 30 min, 37°C) and alkylated (5 mM iodoacetamide, 1 h, 37°C) and then diluted fivefold with 100 mM ammonium bicarbonate, pH 8.0. Sequencing grade-modified trypsin was added (1:30, enzyme:protein) and the samples were incubated for 16 h at 37°C. The resulting peptides were desalted on a PepClean-C18 spin column (Thermo Scientific), normalized by measuring the absorbance at 220 nm (Nanodrop), and fractionated by strong cation exchange (SCX) chromatography. For SCX fractionation, the peptides were bound to a Polysulfoethyl-A microspin TopTip (Glygen) and eluted stepwise with 20-μl volumes of increasing ammonium acetate (20, 40, 60, 80, 120, 160, and 200 mM) in 20% acetonitrile, pH 2.9–3.8 to yield a total of seven fractions. The peptides were then dried (SpeedVac) and reconstituted in 30 μl of 0.1% formic acid/3% acetonitrile. Each fraction was analyzed in triplicate by injecting 7-μl aliquots onto a Biocasic C18 PicoFrit reverse phase nanospray column (74 μm ID × 10 cm, New Objective). Peptides were eluted directly into an LTQ MS (Thermo Scientific) using a 90-min linear gradient of 15%–45% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min. MS spectra were collected over an m/z range of 200–2,000 Da. MS/MS spectra were collected for the five most abundant ions in each MS scan using a dynamic exclusion limit of 2 MS/MS spectra of a given mass for 30 s with an exclusion duration of 10 s.

Fig. 1. Enrichment of γ-glutamyltranspeptidase (γ-GT) activity in isolated brush-border membrane vesicles (BBMV). Crude homogenates of rat renal cortex and of isolated BBMV in the cortex and proximal convoluted tubules (BBMV\textsubscript{CTX} and BBMV\textsubscript{PCT}, respectively) were assayed for γ-GT activity and protein. The fold-increases in specific activity are plotted as a bar graph ± SD for 3 separate samples.
The relative abundance of the identified proteins in the BBMV isolated from cortex (BBMV\textsubscript{CTX}) or from purified proximal convoluted tubules (BBMV\textsubscript{PCT}) was determined by spectral counting (33). Similarity of sampling across injections was examined by comparing the spectral counts (SpC) for each analysis. SpC were also compared by summing each of the SCX fractions from a single sample. Correlation between injections and between each of the biological samples was calculated using the Pearson moment correlation function with the R statistics package (v.2.8.1; http://www.r-project.org). Several diagnostic variables were calculated for each biological sample. These included total spectra, total identified spectra, percentage of total spectra above the search engine threshold cutoffs, and total proteins identified. After a review of these diagnostics, each protein was tested for differential abundance.

Fisher’s exact test (37, 46) was used to identify significant differences between the BBMV\textsubscript{CTX} and BBMV\textsubscript{PCT} samples and to calculate \( P \) values for each protein. Tests were run after summing the SpC for each protein over the biological replicates. The Benjamini-Yekutieli multiple testing adjustment (2) was applied to control the false discovery rate. For each protein, the ratio of the spectral counts (RSC) for each BBMV preparation as an additional threshold for protein identification. Transmembrane domains were predicted using the TMHMM server (v. 20) (http://cbs.dtu.dk) (24, 38).

The relative abundance of the identified proteins in the BBMV isolated from cortex (BBMV\textsubscript{CTX}) or from purified proximal convoluted tubules (BBMV\textsubscript{PCT}) was determined by spectral counting (33). Similarity of sampling across injections was examined by comparing the spectral counts (SpC) for each analysis. SpC were also compared by summing each of the SCX fractions from a single sample. Correlation between injections and between each of the biological samples was calculated using the Pearson moment correlation function with the R statistics package (v.2.8.1; http://www.r-project.org). Several diagnostic variables were calculated for each biological sample. These included total spectra, total identified spectra, percentage of total spectra above the search engine threshold cutoffs, and total proteins identified. After a review of these diagnostics, each protein was tested for differential abundance.

Fisher’s exact test (37, 46) was used to identify significant differences between the BBMV\textsubscript{CTX} and BBMV\textsubscript{PCT} samples and to calculate \( P \) values for each protein. Tests were run after summing the SpC for each protein over the biological replicates. The Benjamini-Yekutieli multiple testing adjustment (2) was applied to control the false discovery rate. For each protein, the ratio of the spectral counts (RSC) for each BBMV preparation as an additional threshold for protein identification. Transmembrane domains were predicted using the TMHMM server (v. 20) (http://cbs.dtu.dk) (24, 38).

\[
R_{SC} = \log_2 \left( \frac{n_{PCT} + 0.5}{n_{CTX} + 0.5} \right) + \log_2 \left( \frac{n_{CTX} - n_{CTX} + 0.5}{n_{PCT} - n_{PCT} + 0.5} \right)
\]
An adjusted $P$ value $\leq 0.001$, a $R_{SC} > 1$, and a minimum of two identified peptides per protein were used as the cutoff for a significant change in protein abundance between the BBMV_{CTX} and BBMV_{PCT} samples. Once significant differences were determined, the $R_{SC}$ values were plotted vs. the total SpC for each protein in the combined BBMV_{CTX} and BBMV_{PCT} samples. The combined cutoff values were then used to visualize the distribution of the proteins that were significantly enriched in either BBMV preparation.

Enrichment of gene functional annotations. A list of Gene Identifier accession numbers corresponding to each IPI accession was created using Microsoft Access software. The list of Gene Identifiers for the two samples (BBMV_{CTX} and BBMV_{PCT}) were then used to upload the proteins into the DAVID 2008 Bioinformatics Resources web server (http://david.abcc.ncifcrf.gov/) for analysis (14, 19). Lists of total proteins per sample were tested for enrichment of functional annotations using the suggestions of Huang et al. (19). Rattus norvegicus was used as the background species to test for enrichment of gene ontology and Kegg pathway terms. Biological process, cellular component, and molecular function were tested separately. Functional annotations were selected based on known inferences of cell function for our samples (i.e., brush-border membrane, proximal tubule function, glycolysis, etc.). Lists of interest were assembled based on these inferences and then expanded by manually evaluating proteins whose functional annotations were incomplete.

RESULTS AND DISCUSSION

Enrichment of BBMV from cortex and from proximal convoluted tubules. Initially, BBMV_{CTX} were prepared by selective MgCl$_2$ precipitation from a homogenate of rat renal cortex (5). Compared with the cortical homogenate, the isolated BBMV_{CTX} exhibit a 15-fold increase in specific activity of $\gamma$-glutamyltranspeptidase, an apical membrane marker for the proximal tubule (Fig. 1). Western blot analysis (Fig. 2A) also indicated that the BBMV_{CTX} are highly enriched in the apical Na$^+$/H$^+$ exchanger (NHE3). However, the BBMV_{CTX} are essentially free of the mitochondrial succinate dehydrogenase (SDH) and CNX but contain reduced levels of GLUT2. These data are in agreement with previous results (5) and are consistent with the removal of other subcellular membranes. However, the isolated BBMV_{CTX} exhibit increased levels of markers for the apical membranes of the collecting duct (aquaporin-2), the thick ascending limb, (Na$^+$/K$^+$/2Cl$^-$/cotransporter-2), and the distal tubule (thiazide-sensitive Na$^+$/Cl$^-$/cotransporter) (Fig. 2B). These data indicate that the classic preparation of BBMV also contains the apical membranes from cortical cells other than the proximal tubule.

![Fig. 4. Comparison of the total spectral counts (SpC) associated with the 20 most abundant proteins identified in the combined BBMV_{PCT} and BBMV_{CTX} samples. SpC determined for each protein in the BBMV_{PCT} and BBMV_{CTX} samples are illustrated by the black bar and the grey bar, respectively.](http://ajprenal.physiology.org/)

<table>
<thead>
<tr>
<th>Protein</th>
<th>BBMV_{CTX}</th>
<th>BBMV_{PCT}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$/K$^+$/-ATPase-1, $\alpha$ subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-density lipoprotein receptor-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHE3 regulatory cofactor 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rBAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipeptidylpeptidase 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malatase-glucoamylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ezrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villin 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$^+$/glucose cotransporter 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$^+$/K$^+$/-ATPase-1, $\beta$ subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type H$^+$/ATPase, subunit B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sushi domain-containing protein 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamyl aminopeptidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\gamma$-glutamyltranspeptidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$^+$/ATPase, V1- A subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$-actinin-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHE3 regulatory factor 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Previous studies indicated that the other cortical cells were effectively removed by using Percoll gradient centrifugation to purify proximal tubules (11, 41). By microscopic inspection, tubules isolated by this procedure appear to be >90% pure proximal convoluted tubules (S1 and S2 segments) with very few proximal straight tubules (S3 segments). The isolated tubules were homogenized and used to isolate BBMVPCT by MgCl2 precipitation. As anticipated, Western blot analysis of the isolated BBMVPCT indicated a significant reduction in the intensity of the apical membrane proteins from non-proximal tubular cells compared with the BBMVCTX (Fig. 2B). As a control, the samples were also probed for NHERF1, an abundant protein that is localized to the apical membrane (42). Interestingly, the specific activity of γ-glutamyltranspeptidase in the BBMVPCT is increased only twofold compared with the cortical homogenate (Fig. 1). Previous enzymatic analysis of dissected tubular segments (9, 10) and immunofluorescence studies (35, 36) indicated that γ-glutamyltranspeptidase is predominantly expressed in the proximal straight tubule of the rat kidney. Therefore, the lower fold-increase in γ-glutamyltranspeptidase activity is a further indication that the BBMVPCT are derived largely from the proximal convoluted tubule. A comparative proteomic analysis of BBMVCTX and BBMVPCT samples should verify the conclusions that the latter preparation is more highly enriched and is derived specifically from the proximal convoluted tubule.

Proteomic analysis of BBMVCTX and BBMVPCT. Duplicate biological samples of BBMVCTX and BBMVPCT were analyzed by two-dimensional LC-MS/MS. Each sample was initially separated by off-line SCX chromatography to produce seven fractions. Triplicate samples from each of the SCX fractions were further fractionated by online C18-chromatography coupled directly to an LTQ MS via a nanoelectrospray source. The SEQUEST and X!Tandem search engines were used to match the recorded MS/MS spectra from the 84 MS analyses against the current rat IPI database. The resulting identifications were validated in Scaffold using stringent criteria that produced a false discovery rate of ≤1%. This analysis

![Fig. 5](http://ajprenal.physiology.org/)

**Fig. 5.** Scatterplots of total SpC in replicate samples. *A*: correlation of total SpC between replicate injections. Pearson’s product indicated a correlation of $r = 0.97$ between typical examples of replicate injections. *B*: correlation of total SpC between biological replicates. Pearson’s product indicated correlations of $r = 0.94$ and $r = 0.95$ between the replicate BBMVCTX and BBMVPCT samples, respectively.
identified 193 proteins common to both preparations of BBMV. 57 proteins unique to the BBMV_PCT, and 21 proteins unique to the BBMV_CTX (Fig. 3A). Functional analysis of the 250 proteins contained in the BBMV_PCT indicated that nearly 30% of the identified proteins are transporters, peptidases, and cytoskeleton proteins that are typical of the brush-border membrane (Fig. 3B). An additional 12% of the proteins are membrane receptors and proteins involved in signal transduction. Of the total identified proteins, 22% are integral membrane proteins and an additional 18% are peripheral membrane proteins. However, the single largest group of identified proteins is those involved in metabolism. Functional analysis of the 214 proteins identified in the BBMV_CTX and BBMVPCT samples was performed to verify the differences in the two preparations.

Quantitative analysis of protein abundance in BBMV_CTX and BBMV_PCT. SpC collected for an individual protein increase in proportion to its abundance (33). As a result, spectral counting has become the preferred method of quantification due to its ease of implementation compared with alternative methods that require chemical modification of peptides or labeling with heavy isotopes. However, to utilize the SpC to quantify the differences in protein abundance, the data derived from each sample must be carefully analyzed to ensure that no significant loss of proteins or peptides occurred during the sample preparation. For the reported data, the SD of the total identified SpC for each of the four biological samples expressed as a percentage of the mean was only 5.6%. The same analysis for total spectra (unidentified and identified), percentage of total spectra identified, and the number of protein identifications yielded values of 2.6, 6.5, and 7.5%, respectively. The highest variance was between the two BBMV_CTX samples. This could be due to the heterogeneity introduced by the dissection of the renal cortex. To further assess the consistency of replicate samples, the total SpC for each of the identified proteins was compared between individual injections (Fig. 5A) and between the sum of all injections (Fig. 5B). As expected, the greatest dissimilarity was observed in the SpC measured for the less abundant proteins. However, the data had Pearson's correlation coefficients of \( r = 0.97 \) between representative injections and \( r = 0.94 \) and \( r = 0.95 \) for the replicate BBMV_CTX and BBMVPCT samples, respectively. Therefore, the SpC measured in the multiple injections and the biological replicates were highly reproducible.

Fisher's exact test (37) was used to identify significant changes in protein abundance between the BBMV_CTX and BBMVPCT samples. The resulting \( P \) values were adjusted for multiple testing using the Benjamini-Yekutieli correction (2). The criteria for identifying a significant change were set as RSC > 1 (i.e., >2-fold change in total SpC) and a corrected \( P \) value of ≤ 0.001. The normalized RSC for the 271 proteins identified in the two BBMV samples were plotted vs. the total SpC and color coded to identify proteins that are significantly
enriched in either BBMV preparation (Fig. 6). This analysis identified 42 proteins that were significantly enriched in the BBMVCTX and 50 proteins enriched in the BBMVPCT. The proteins that are significantly enriched in the two samples are italicized in Supplemental Table S1.

The compiled data revealed significant enrichment of several interesting proteins in both the BBMVCTX and BBMVPCT preparations. Functional analysis of the data indicates that membrane transporters constitute 45% of the proteins enriched in the BBMVCTX (Fig. 7A), but only 21% of the total proteins identified in the BBMVCTX (Fig. 3C). Some of the proteins that are significantly enriched in the BBMVCTX are illustrated in Fig. 8. The $R_{sc}$ values are the estimated log$_2$ ratios of abundance comparing BBMVCTX to BBMVCTX. Included in this list are the thiazide-sensitive Na$^+$Cl$^-$ cotransporter and Na$^+$K$^+$2Cl$^-$ co-transporter-2 that are derived from the apical membranes of the distal tubule and the thick ascending limb, respectively. These data are validated by the previous Western blot analysis (Fig. 2B) and strongly support the conclusion that the BBMVPCT are essentially free of contaminating apical membranes from non-proximal tubule cells. The BBMVCTX samples are also significantly enriched for meprin, nephrilysin, the amino acid transporter rBAT, glutamine synthetase, and γ-glutamyltranspeptidase. Of particular interest is the identification of nephrilysin, which is not expressed in the S1 and S2 segments of the proximal tubule (34, 40). In addition, rBAT (17), glutamine synthetase (7), and γ-glutamyltranspeptidase (35) are proteins that are primarily expressed in the S3 segment. The increases in rBAT and γ-glutamyltranspeptidase quantified by spectral counting were confirmed by Western blot analysis (Fig. 9). Thus the observed increase in their abundance in the BBMVPCTX further validates the conclusion that the BBMVPCT are derived primarily from the proximal convoluted tubule.

Functional analysis of the proteins that were enriched in the BBMVPCT group revealed an increased abundance of enzymes of metabolism and mitochondrial proteins (Fig. 6B). The former group constitutes 38% of the proteins that are significantly enriched, but only 19% of the total proteins identified in the BBMVPCT (Fig. 3B). This group contains 11 proteins (Supplemental Table S1) involved in glucose metabolism including aldolase B and fructose 1,6-bisphosphatase-1 (Fig. 8). Aldolase B was previously reported to colocalize with the vacuolar H$^+$/ATPase in the apical membrane and the submicr villar zone of endocytic vesicles (27). This interaction is essential for the assembly and the activity of the proton pump (26, 28). Immunofluorescence studies demonstrated that fructose 1,6-bisphosphatase-1 is also concentrated in the apical region of the rat renal proximal convoluted tubule (44). As a result of their localization, aldolase B and fructose 1,6-bisphosphatase-1 may be trapped in the BBMVPCT that are formed during homogenization of isolated proximal convoluted tubules. The enrichment of multiple enzymes of glucose metabolism also supports the concept that the enzymes form a complex that facilitates the channeling of intermediates of glucose metabolism.
glycolysis and/or gluconeogenesis (32). The positioning of this complex adjacent to the brush-border membrane may facilitate the catabolism of glucose that is reabsorbed from the glomerular filtrate. Alternatively, following an overnight fast (18) or the onset of acidosis (20), this complex could facilitate the net renal synthesis of glucose from reabsorbed gluconeogenic precursors.

Mitochondrial proteins constitute 22% of the proteins that are enriched in the BBMV<sub>PCT</sub> but only 11% of total identified proteins. The proteins enriched in the BBMV<sub>PCT</sub> included components of the mitochondrial ATP synthase, including the α-, β-, and oligomycin-binding subunits (Fig. 8). The mitochondria within the proximal convoluted tubule are larger, more elongated, and more abundant than those within the proximal straight tubule (23). In addition, the mitochondria in the S1 and S2 segments are positioned in close proximity to the well-developed endocytic apparatus immediately underneath the brush-border membrane. By contrast, the small circular mitochondria found in the S3 segment are more evenly distributed throughout the cytosol. As a result, mitochondrial proteins, released during homogenization, may be preferentially trapped in the BBMV<sub>PCT</sub>.

In summary, the reported data establish that sequential isolation of proximal tubules by Percoll gradient centrifugation and MgCl<sub>2</sub> aggregation yields a highly enriched preparation of BBMV that are derived preferentially from the S1 and S2 segments of the proximal convoluted tubule. Comparison of the reported proteomic data to those of Cutillas et al. (12) also indicates that this preparation contains a similar level of intracellular contaminants as observed with BBMV purified by capillary free-flow electrophoresis. However, the reported protocol is simpler and utilizes equipment that is more generally available. Thus the reported protocol and database provide the basis for a comprehensive analysis of the temporal changes in the brush-border membrane that occur in response to various physiological adaptations or pathological conditions.

**REFERENCES**

24. Iynedjian PB, Ballard FJ, Hanson RW. The regulation of phosphoenolpyruvate carboxykinase (GTP) synthesis in rat kidney cortex. The role of