Purification and Partial Characterization of Candidate Antidiuretic
Hormone Water Channel Proteins of M, 55,000 and 53,000 from
Toad Urinary Bladder*

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Antidiuretic hormone (ADH) increases toad bladder granular cell apical membrane osmotic water permeability ($P_w$) by insertion of cytoplasmic vesicles containing water channels into the apical membrane. Termination of ADH stimulation results in endocytosis of water channel-containing membrane. In previous work, we have purified water channel-containing vesicles and demonstrated that they contain 12 major protein bands when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). On the basis of vectorial labeling studies of granular cells and purified vesicles, we have proposed previously that vesicle proteins of M, 55, and 17 kDa are ADH water channel components. In this report, we have purified and analyzed these three proteins using a combination of SDS-PAGE, peptide mapping, amino acid composition, and amino-terminal analyses. The 55- and 53-kDa proteins are distinct protein species possessing a high degree of structural similarity. Both possess a large content of cysteine. The 17-kDa protein appears to be a proteolytic fragment of the 53-kDa protein. None of these three proteins is phosphorylated or contains large amounts of covalently linked carbamidomethyl. ADH-elicted $P_w$ is inhibited by the organic mercurial reagent fluorescein mercuric acetate (FMA). Exposure of water channel-containing vesicles to FMA labels selectively four vesicle proteins of 92, 55, 53, and 29 kDa while reducing vesicle $P_w$ by 82%. The combination of FMA and 2-mercaptoethanol or exposure to another mercurial reagent, N-ethylmaleimide, does not inhibit vesicle $P_w$. Together, these data provide additional evidence for the role of the 53- and 55-kDa proteins as components of the ADH water channel. These candidate ADH water channel proteins are distinct from a 28-kDa candidate water channel protein (CHIP 28) isolated recently from human erythrocyte membranes and kidney proximal tubule by Agre and co-workers (Preston, G. M., Carroll, T. P., Guggino, W. B., and Agre, P. 1992 Science 256, 385-387).

ADH-stimulated insertion of water channels in toad urinary bladder shows they are highly selective and are permeable to both water (4-6) and protons (7-10) while excluding larger ions. Water channel $P_w$ is inhibited by exposure to organic mercurial compounds such as p-chloromercuribenzenesulfonate (p-CMBS) and fluorescein mercuric acetate (FMA) (11-13). Mercurial inhibition of water channel $P_w$ may be prevented by simultaneous addition of sulfhydryl reducing compounds.

ADH-mediated insertion of water channels has been characterized extensively in granular cells of the toad urinary bladder. Ultrastructural studies using freeze fracture electron microscopy have related water channel insertion to the appearance of structures called particle aggregates in the apical membrane of ADH-responsive cells (14, 15). Prior to ADH stimulation, apical membrane $P_w$ is low and water channels are stored in dense arrays in the limiting membranes of large cytoplasmic vesicles called aggrephores (14-16). ADH stimulation causes aggrephores to fuse with the granular cell apical membrane and increase $P_w$. Removal of ADH decreases $P_w$ and water channels are retrieved into the cytoplasm via apical membrane endocytosis (17-19). Comparison of SDS-PAGE protein bands labeled with lactoperoxidase in either unstimulated or ADH-stimulated granular cell apical membranes has suggested that protein bands of M, 55, 17, 15, and 7 kDa are ADH water channel components (20). Similar results have been obtained after labeling membranes with the fluorescent membrane-impermeant reagent eosin maleimide (21), fractionation of purified ADH-stimulated apical membrane by two-dimensional gel electrophoresis (22), or from studies using polyclonal antisera raised against these proteins (23, 24).

In previous work, we have purified water channel-containing vesicles (WCV) from granular cell homogenates (6) using either density shifting (25) or flow sorting (26) techniques. SDS-PAGE analysis of purified WCV demonstrates they possess 12 major protein species. Three protein bands of M, 55, 53, and 17 kDa constitute 55% of the total protein content of

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Characterization of Candidate ADH Water Channel Proteins

Phenylmethylsulfonyl fluoride, diaminobenzidine (DAB), parachloromercuribenzenesulfonate (p-CMBS), fluorescein mercuric acetate (FMA), N-ethylmaleimide (NEM), and Tween 20 were purchased from Sigma. Coomassie Blue R-250, Amido Black, Triton X-100, and SDS-PAGE reagents were obtained from Bio-Rad. We utilized Crotex Lightning-Plus enhancing screens from Du Pont and cellulose nitrocellulose and gelatin were from J. T. Baker Inc. Nitrocellulose and membranes were from Schleicher and Schuell. Stained SDS-PAGE gels were dried on a heated gel dryer for autoradiography or selected portions of protein bands excised from PVDF blots and either stained with 0.1% Amido Black and destained with 50% methanol, 45% water, 5% acetic acid mixture or stained with 0.1% Coomassie Blue R-250, or gel proteins were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes. SDS-PAGE gels containing vesicle proteins were excised from the PVDF blot and treated with n-tetramethylammonium hydroxide in 6 N HCl. For each sample, an identical size PVDF membrane was prepared and oxidized with nitroblue tetrazolium. Nitrocellulose-bound proteins were oxidized with oxidized-digoxigenin-succinyl-maleimido-glucosamine conjugates. Reactivity of 55-, 53-, and 17-kDa protein bands were assessed by the method of O'Shannessy and Boulanger (34) using reagents provided by the Glycan detection kit (Boehringer Mannheim). Nitrocellulose-bound proteins were exposed to ethanolic sodium metaperiodate, washed, and reacted with digoxigenin-succinyl-amidoacaproic acid hydrazide. Digoxigenin-labeled glycoconjugates were subsequently detected by enzyme-linked immunooassay using alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments. Reactivity of 55-, 53-, and 17-kDa protein bands were compared with that exhibited by the glycoprotein transferrin assayed under identical conditions.

Peptide Mapping of 55-, 53-, and 17-kDa Proteins—Peptide mapping analyses were carried out as described by Elder et al. (35) using plastic-backed cellulose thin layer plates on a flat bed electrophoresis unit (Savant Inc., Hicksville, NY). When the peptide compositions of two plates were being compared, they were electrophoresed and chromotographed together. All autoradiography was carried out at −60 °C using enhancing screens. Peptide mobilities were determined by multiple autoradiography exposures and measurements of the mobility of each peptide with respect to the origin.

Amino Acid and Amino-terminal Analyses—Excised protein bands fixed on PVDF membranes were rinsed briefly in 20% methanol, 80% water, then hydrolyzed at 105 °C for 24 h in vacuum-sealed glass vials containing 6 N HCl. For each sample, an identical size PVDF membrane containing the same blot containing no stainable protein was included as a control as described by LeGendre and Matsudaira (36). Preliminary studies using purified proteins (β-galactosidase and albumin) demonstrated that transfer and hydrolysis on PVDF membranes did not produce any significant changes in their amino acid composition (data not shown). These data are consistent with more extensive studies performed on a wide variety of PVDF bound proteins by Tarr et al. (36).

Protein bands excised from PVDF blots were also subjected to amino-terminal sequence analysis using an Applied Biosystems model 4770 protein sequenator.

Analysis of FMA-derivatized WCV Proteins—Analysis of FMA-derivatized WCV proteins was performed as described above, then purified by density shifting on WCV-P. The effect of preincubation of mercurial reagents on WCV-P was quantitated by monitoring the fluorescence quenching of entrapped carboxyfluorescein (CF) as described previously (5, 6, 10). Briefly, WCV were loaded with 10 mM CF in a fashion identical to that described for horseradish peroxidase. A partially purified membrane fraction (intermediate pellet) containing CF-loaded WCV was used for all studies. All P/ measurements were performed using an Applied Photophysics SF017 stopped flow fluorimeter with a measured dead time of 0.7 ms. Mean values of P/ were expressed as cm/s ± standard error. The statistical significance of differences between groups of P/ measurements were tested using the Bonferroni t test (37).

Inhibition and labeling studies were performed on vesicles resuspended in 500 μl of homogenization buffer (2.5 mM HEPES, 7.5 mM KCl, and 0.1 mM EDTA, pH 8.0) and preincubated for 30 min with either 2 mM p-CMB, 0.25 mM FMA, or 0.5 mM NEM prepared as 4, 0.5, and 5 mM stocks, respectively, in homogenization buffer immediately prior to use. In selected experiments, 2-mercaptoethanol was added, to a final concentration of 2 mM, 5 min prior to FMA addition. After these incubations were completed, the vesicles were collected by centrifugation at 10,000 × g for 10 min, washed twice in homogenization buffer and 100 μl of anti-fluorescein antisera (described in detail below) added to quench all extravesicular fluorescence. WCV-P was then measured using stopped flow fluorimetry.

Analysis of FMA-derivatized WCV Proteins on Western Blots—Horse radish peroxidase-loaded WCV were incubated with FMA as described above, then purified by density shifting. WCV proteins were treated with SDS-PAGE substituting NEM in place of mercaptoethanol in SDS-PAGE denaturation buffer to prevent detachment of mercurials from proteins (38). Proteins were transferred to nitrocellulose membranes and either stained with 0.1% Amido Black and dilution of 50%, stained with 0.1% p-CMB, or acidified with 20% water, 5% acetic acid and used for immunohosting as described below. In the presence of DAB interferes with standard protein assays, the protein load on each lane was standardized by first staining a separate lane loaded with an identical quantity of density-shifted protein. To quantitate the distribution of blot protein, filters were incubated in 100% Triton X-100 followed by quantitated using a Zeineh soft laser densitometer (Biomed Instrument Co., Fullerton, CA) as described previously (27).
Characterization of Candidate ADH Water Channel Proteins

For identification of FMA proteins, filters containing identical quantities of vesicle protein were subjected to immunoblot analysis using affinity-purified rabbit anti-fluorescein antisera. This was prepared by repeated intradermal immunization of fluorescein-conjugated keyhole limpet hemocyanin in rabbits. The resulting antisera was affinity-purified using an alkaline phosphatase-conjugated affinity-purified goat anti-rabbit secondary antibody and exposure to color development substrates.

RESULTS

Purification of 55-, 53-, and 17-kDa Proteins—Previous data produced from LPO-mediated apical membrane and intraepithelial [3H]-labeled ADH-stimulated granular cells (20) as well as quantitation of proteins from purified WCV (27) revealed that candidate ADH water channel proteins of 55 and 53 kDa appear as distinct sharp bands, while the 17-kDa band is quite broad, stains poorly with Coomassie Blue, and extends from 20 to 15.5 kDa. To obtain sufficient quantities of purified material to examine the structure and composition of these proteins, we isolated WCV and subjected pooled vesicle fractions to preparative SDS-PAGE to purify these protein bands. Fig. 1 displays representative gels containing protein bands of 55 and 53 kDa (lane A) and 17 kDa (lane B) used for analytical studies described below. Optimal separation of the 55- and 53-kDa proteins was obtained using 9% acrylamide gels under conditions where the bromphenol blue dye front was permitted to run off and electrophoresis continued until proteins possessing molecular masses of 28 kDa were present in the dye front of the gel. The 17-kDa protein was purified using 12% SDS-PAGE gels. As reported previously (27), the 55- and 53-kDa bands were present in a 2:1 ratio. In contrast to the 55- and 53-kDa bands, the 17-kDa protein band stained faintly with Coomassie Blue. Three hundred micrograms of 55- and 53-kDa protein bands were prepared representing approximately 5 nmol of each protein. Eight micrograms of 17-kDa protein band (approximately 4.7 nmol) was prepared in an identical fashion. To prevent possible cross-contamination of the 55- or 53-kDa protein by its neighboring band, only the upper (55 kDa) or lower (53 kDa) half of the stained protein band was used for peptide mapping, compositional, or amino-terminal analyses described below.

The 55-, 53-, or 17-kDa Bands Do Not Contain Significant Quantities of Covalent Exchangeable Phosphate or Carbohydrate—It is possible that the 55/53-kDa doublet or the broad 17-kDa band may be due in part to post-translational modifications such as phosphorylation or covalent carbohydrate addition. In previous studies (40), we have demonstrated that the 17-kDa protein is not [32P]-labeled after incubation of intact toad bladders with [32P]orthophosphate. To establish whether the 55- or 53-kDa proteins contain significant quantities of exchangeable phosphate, intact toad bladders were incubated with [32P]orthophosphate under identical conditions and [32P]content of the 55- and 53-kDa bands assessed by identified autoradiography of gels identical to that shown in Fig. 1. Fig. 2 shows that neither the 55- nor the 53-kDa band is [32P]-labeled.

To assess whether the 55-, 53-, or 17-kDa protein species contains significant quantities of covalent carbohydrate, bands on nitrocellulose were subjected to mild periodate treatment with subsequent attachment of digoxigenin via a hydrazide group. Digoxigenin-labeled glycoconjugates were then detected using alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments as described under "Experimental Procedures." None of the three protein bands demonstrated significant reactivity under conditions where covalent carbohydrate was readily detected in 1 μg of transferrin (Fig. 3). On the basis of these data, it appears that the 55-, 53-, or 17-kDa protein bands do not contain significant quantities of exchangeable or covalent carbohydrate.

Peptide Mapping of the 55-, 53-, and 17-kDa Protein Bands—We used two-dimensional mapping of chymotryptic peptides derived from fixed, Coomassie Blue-stained protein bands to determine the structural relationships between the 55-, 53-, and 17-kDa protein bands. This method has been widely used to determine relationships between purified SDS-PAGE protein bands (29, 41-43). Fig. 4 contains representative maps that compare the distribution of [32P]-labeled chymotryptic peptides from the 55- and 53-kDa (upper panel) and 17-kDa (lower panel) protein bands. The peptide maps of the 55- and 53-kDa bands are similar but not identical. Detailed comparisons of these maps reveal the presence of many common peptides in addition to the presence of a limited number of peptides that are unique to either the 55- or 53-kDa band. All of the major chymotryptic peptides of the 17-kDa protein have counterparts in the maps of the 55- and 53-kDa proteins (Fig. 4, lower panel). Together, these...
Characterization of Candidate ADH Water Channel Proteins

Fig. 3. 55- and 53-kDa proteins do not contain significant quantities of covalently linked carbohydrate. The right panel (Protein) shows 40 μg of WCV proteins (lane 1) and 1 μg of transferrin (lane 2) stained with Coomassie Blue. The left panel (Carbohydrate) shows identical quantities of transferrin (lane 3) and WCV proteins (lane 4) after mild periodate treatment and detection of covalently linked carbohydrate via a digoxigenin-linked alkaline phosphatase system (see "Experimental Procedures"). Note that while both transferrin and several other WCV proteins appear as carbohydrate containing bands in this assay, the 55- and 53-kDa proteins (shown by the asterisk) do not. The fainter bands above and below transferrin are presumably complexes and fragments of transferrin present in this purified commercial source.

The data suggest that the 55- and 53-kDa proteins possess similar structural characteristics and the 17-kDa protein band is possibly a fragment or subunit derived from the 55- and 53-kDa proteins.

Amino Acid Compositions and Amino-terminal Analyses of 55, 53, and 17-kDa Protein Bands—Table I lists the average of three compositional analyses for each of the three proteins expressed as mol %. Comparison of the 55- and 53-kDa proteins reveal they have very similar compositions. There are, however, notable differences between the 55- and 53-kDa species. These include the 53-kDa protein's higher content of glutamate/glutamic acid (Glx; 12.1 mol %) as compared with that of the 55-kDa protein (2.1 mol %). The 55-kDa protein contains nearly twice as much tyrosine as does the 53-kDa band. Interestingly, both proteins are highly enriched in cysteine and contain little proline.

The composition of the 17-kDa protein band is notable for its lack of histidine, threonine, tyrosine, lysine, or proline. It contains 3.5 mol % cysteine and is enriched for leucine, glycine, alanine, and Gl(x). These compositional data provide additional support for the hypothesis that the 17-kDa band may derive from either the 55- or 53-kDa proteins. The 17-kDa protein contains 11.6 mol % or approximately 17 residues of Gl(x). Since the Gl(x) content of the 17-kDa protein exceeds the Gl(x) content of the 55-kDa band (2.1 mol % or 10 Gl(x) residues), the 17-kDa protein may actually derive from the 53- rather than the 55-kDa protein. The larger Gl(x) content (12.1 mol %) of the 53-kDa protein could allow the 17-kDa protein to derive from it by proteolysis. If the 17-kDa protein is indeed a fragment of the 53-kDa protein, these data may suggest that the bulk of the Gl(x) residues are clustered within one region of the 53-kDa polypeptide.

The data contained in Table II show that the amino termini of the 55- and 53-kDa proteins appear to be blocked. In contrast, identical analysis of the 17-kDa protein yields multiple amino-terminal amino acids including glycine, asparagine, and valine. Previously, we have observed that exclusion of the protease inhibitors phenylmethylsulfonylfluoride, leupeptin, and EDTA results in lower yields of the 55- and 53-kDa protein bands in conjunction with increased staining of the 17-kDa protein band (27). Thus, these data are consistent with the possibility that the multiple amino termini displayed by the 17-kDa protein band may result from proteolysis.

FMA and p-CMBS Both Inhibit WCV P.—Although the 55- and 53-kDa proteins are abundant in WCV (27) and membrane labeling studies (20, 23, 24) suggest strongly that these integral membrane proteins may be water channel components, there has been no evidence linking directly the modification of these proteins to functional changes in ADH water flow. Since affinity-purified rabbit anti-fluorescein antisera binds to the fluorescein moiety of fluorescein mercuric acetate (FMA), an inhibitor of ADH water flow in anuran bladders (12, 13), we attempted to link FDA induced inhibition of water channels in WCV with the specific covalent labeling of the 55-, 53-, or 17-kDa proteins. Fig. 5 shows the effects of preincubation with either p-CMBS (lower panel) or FMA (upper panel) on water channels present in WCV. WCV were exposed to either p-CMBS or FMA in a series of paired experiments. The middle panel demonstrates that control
Protein bands (see Fig. 1) were transferred to PVDF membranes, visualized by Coomassie Blue staining, excised, and hydrolyzed in 6 M HCl at 107 °C for 24 h. Their amino acid composition was determined on a Hewlett-Packard model 1090 Aminoquant system. GI(x) and As(x) refer to Glu/Gln or Asp/Asn, respectively. Tryptophan was not determined. Values are the average of three independent analyses. All values are expressed in mol % and calculated after subtraction of any amino acids present on a PVDF blank membrane.

**Table I**

Amino acid composition of 55-, 53-, and 17-kDa vesicle proteins

A total of 1.5, 0.75, and 0.80 nmol of purified 55-, 53-, and 17-kDa protein bands (see Fig. 1) were transferred to PVDF membranes, visualized by Coomassie Blue staining, excised, and hydrolyzed in 6 M HCl at 107 °C for 24 h. Their amino acid composition was determined on a Hewlett-Packard model 1090 Aminoquant system. GI(x) and As(x) refer to Glu/Gln or Asp/Asn, respectively. Tryptophan was not determined. Values are the average of three independent analyses. All values are expressed in mol % and calculated after subtraction of any amino acids present on a PVDF blank membrane.

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<th>53 kDa</th>
<th>17 kDa</th>
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<td>11.4</td>
<td>5.8</td>
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<tr>
<td>Gl(x)</td>
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<tr>
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</tr>
<tr>
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<table>
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<tr>
<td>17 kDa*</td>
<td>Cycle 3 31 pmol Glu, 11 pmol Leu, 8 pmol Ser</td>
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* Yields from representative sequence analysis cycles 2 and 3 are displayed. Cycle 1 was not informative.

WCV were highly water-permeable (P_f = 0.115 ± 0.017 cm/s; n = 23) as described previously (10). Preincubation with 1 mM p-CMBS resulted in a 93% reduction of WCV P_f to a value of 0.008 ± 0.0025 cm/s (n = 9; P < 0.001). In a similar fashion, preincubation with 0.25 mM FMA reduced WCV P_f by 82% to a value of 0.021 ± 0.005 cm/s (n = 9; P < 0.001). Inhibition of WCV P_f by p-CMBS and FMA appeared to be specific for mercurials and mediated through binding to sulfhydryl groups. Exposure to 0.5 mM n-ethylmaleimide (NEM) did not affect WCV P_f (0.115 ± 0.017 cm/s; n = 23). Preincubation for 30 min with either 0.25 mM FMA (upper panel) or 1 mM p-CMBS (lower panel) caused a 82% (0.021 ± 0.005 cm/s; n = 9) and 93% (0.008 ± 0.0025 cm/s; n = 9) reduction in WCV P_f, respectively. Note the different time scales of each panel. A total of 400 data points were obtained in each panel with one-half recorded during the initial change in vesicle volume (darker portion of tracings) to enhance the determination of initial rates.

The **lower panel** of Fig. 6 shows a representative Western blot from these seven experiments. WCV proteins labeled by FMA exposure, since gels from control (50 ± 5%) and FMA-treated (48.5 ± 6%) WCV were indistinguishable. The **lower panel** of Fig. 6 shows a representative Western blot from these seven experiments. WCV proteins labeled by FMA exposure, since gels from control (50 ± 5%) and FMA-treated (48.5 ± 6%) WCV were indistinguishable.

**Discussion**

Based on a combination of data we have proposed previously that the 55- and 53-kDa proteins present in WCV are...
characterization of candidate ADH water channel proteins.

Table I shows that the 55-, 53-, and 17-kDa proteins possess 11.2, 8.3, and 3.5 mol % cysteine, respectively. Since the recovery of cysteine after acid hydrolysis is rather poor (36), these values likely represent underestimates of the actual cysteine content of these proteins. Since the 55- and 53-kDa proteins are labeled by FMA under conditions that inhibit WCV \( P_f \) (Fig. 5), modification of any of these cysteines may account for the mercurial sensitivity of the ADH water channel. The exact role of these abundant cysteines in water channel function is unknown currently. It is of interest, however, that Type II Arctic fish glycoproteins contain 8% cysteine and, in a fashion similar to that of the ADH water channel, are inactivated by mercurial exposure (44). It is postulated that these proteins of artichoke order water molecules on their surfaces to prevent ice crystal formation. We speculate that the abundant cysteine content of the 55-, 53-, and 17-kDa proteins of WCV may also reflect their functional interactions with water molecules.

Sulfhydryl reactive compounds have been useful in characterizing water transport pathways in the red cell (38), kidney proximal tubule, and amphibian urinary bladder (1, 11). Ibarra et al. (13) showed that FMA inhibits ADH-stimulated \( P_f \) in intact amphibian bladders but only when FMA addition occurs after ADH stimulation. Addition of FMA or p-CMBS to unstimulated bladders does not reduce transepithelial \( P_f \) since water channel proteins are "protected" from the action of these mercurials as they are located in aggregates in the granular cell cytoplasm. FMA and p-CMBS inhibition is reversed by addition of cysteine. These inhibitory effects appear specific since other sulfhydryl reactive compounds such as NEM and iodoacetamide do not mimic p-CMBS or FMA inhibition (45). Data reported here confirm and extend these observations by demonstrating that both FMA and p-CMBS inhibit \( P_f \) in WCV retrieved from the apical membrane of ADH-stimulated bladders. These reagents appear to interact with sulfhydryl groups in labeling WCV proteins of 92, 55, 53, and 30 kDa. Inclusion of 2-mercaptoethanol ablates the inhibition of WCV \( P_f \) by FMA and NEM has no effect on WCV \( P_f \) (see above). These data provide further evidence that the 55- and 53-kDa proteins are components of the ADH water channel by linking the labeling of these proteins to FMA-induced inhibition of WCV \( P_f \).

The 55, 53, and 17-kDa WCV proteins reported here appear to be distinct from a 28-kDa candidate water channel protein designated CHIP 28, which is present in both human erythrocyte membranes and renal proximal tubules and has been characterized recently by Agre and co-workers (28–31). Although the 55-, 53-, and CHIP 28 proteins are all integral membrane proteins, there are several notable differences that distinguish the 55- and 53-kDa WCV proteins from CHIP 28 besides differing molecular masses. These include the following. 1) The 55/53-kDa proteins stain prominently with Coomassie Blue (Fig. 1), whereas the CHIP 28 protein stains poorly with Coomassie Blue as reported by Agre (28, 29). 2) CHIP 28 contains significant quantities of covalent carbohydrate; the 55/53-kDa proteins do not. 3) The 55/53-kDa proteins possess a different amino acid composition than CHIP 28 (notably that CHIP-28 contains only 0.3 mol % cysteine compared with an average of 9 mol % for the 55/53-kDa proteins). Finally, the 55- and 53-kDa candidate ADH water channel proteins should be localized to the ADH-responsive collecting tubules of the mammalian kidney (1). Immunocytochemistry studies (29) using anti-CHIP 28 anti-

Fig. 6. Identification of FMA-labeled vesicle proteins by Western blotting. SDS-PAGE and immunoblotting analysis of FMA derivatized WCV proteins. Upper panel, WCV were exposed to FMA under conditions shown in Fig. 4 (upper panel), purified by density shifting, fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and stained with Amido Black. Lane A shows proteins from control WCV as compared to those from FMA-derivatized WCV (lane B). We observed no significant changes in the overall pattern of WCV proteins. Lower panel, FMA-derivatized WCV proteins were detected by the binding of affinity-purified anti-fluorescein antisera in combination with an alkaline phosphatase conjugated secondary antibody. The left lane (PROTEIN) shows the amount of Amido Black-stained WCV protein used in the immunoblot shown in the right lane (FMA). Note that of the multiple protein bands displayed on the left, only four bands of 92, 55, 53, and 29 kDa bind antifluorescein antisera as indicated by the leftward-facing arrowheads. The position of molecular mass markers is indicated by the smaller rightward-facing arrowheads.

Components of the ADH water channel (1). These data include the fact that the 55-, 53-, and 17-kDa proteins are labeled exclusively by radioactively labeled membrane reagents in the apical membranes of ADH-stimulated granular cells and not in unstimulated water permeable bladders (20). These same proteins represent one-half of the total protein in purified WCV (27), and enabling calculations of the number of ADH water channels necessary to account for the high WCV \( P_f \) suggest that water channels be present at very high densities (1). Detailed study of the topography of WCV membrane proteins demonstrates that the 55- and 53-kDa proteins are integral membrane proteins that span the lipid bilayer of WCV and thus conform to a structure expected for components of a water channel (27).

In this report we have purified each of the 55-, 53-, and 17-kDa proteins and assayed their content of exchangeable phosphate, carbohydrate, and amino acids. None of the three proteins contains significant amounts of exchangeable covalently bound phosphate (Fig. 2) or carbohydrate (Fig. 3). The combination of peptide mapping data and amino acid compositional analyses shows that the 55- and 53-kDa proteins appear to be distinct proteins that share a high degree of structural similarity. Based on topological (27), peptide mapping (Fig. 4), compositional, and amino-terminal analysis (Tables I and II) data, we speculate that the 17-kDa polypeptide may derive from the 53-kDa protein by proteolysis. However, definitive assignment of the origin of the 17-kDa protein must await protein sequence and molecular cloning data.
serum localizes this protein to membranes of the proximal tubule and thin limbs of Henle but not the cortical or medullary collecting tubules. Together, these data suggest that CHIP 28 is not a component of the ADH water channel.

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