

# Cloning, Functional Analysis and Cell Localization of a Kidney Proximal Tubule Water Transporter Homologous to CHIP28

Rubin Zhang, William Skach,\* Hajime Hasegawa, Alfred N. van Hoek, and A. S. Verkman

Departments of Medicine and Physiology, Cardiovascular Research Institute, and \*Cancer Research Institute, University of California, San Francisco, California 94143-0532

**Abstract.** The localization and transporting properties of a kidney protein homologous to human erythrocyte protein CHIP28 was evaluated. The cDNA encoding rat kidney protein CHIP28k was isolated from a rat renal cortex cDNA library. A 2.8-kb cDNA was identified which contained an 807 bp open reading frame encoding a 28.8 kD protein with 94% amino acid identity to CHIP28. *in vitro* translation of CHIP28k cDNA in rabbit reticulocyte lysate generated a 28-kD protein; addition of ER-derived microsomes gave a 32-kD transmembrane glycoprotein. Translation of truncated RNA demonstrated glycosylation of residue Asn42 which is predicted to lie between the first and second transmembrane domains. Expression of *in vitro* transcribed mRNA encoding CHIP28k in *Xenopus* oocytes increased oocyte osmotic water permeability ( $P_f$ ) from  $(4 \pm 1) \times 10^{-4}$  to  $(33 \pm 4) \times 10^{-4}$  cm/s at 10°C; the increase in oocyte  $P_f$  was weakly temperature dependent and inhibited by HgCl<sub>2</sub>. Two-electrode voltage clamp measurements indicated that CHIP28k was not

permeable to ions. Oocyte  $P_f$  also increased with expression of total mRNA from kidney cortex and papilla; the increase in  $P_f$  with mRNA from cortex, but not kidney papilla, was blocked by coinjection with excess antisense CHIP28k cRNA. *In situ* hybridization of a 150 base cRNA antisense probe to tissue sections from rat kidney showed selective CHIP28k localization to epithelial cells in proximal tubule and thin descending limb of Henle.  $P_f$  in purified apical membrane vesicles from rat and human proximal tubule, and in proteoliposomes reconstituted with purified protein, was very high and inhibited by HgCl<sub>2</sub>; stripping of apical vesicles with N-lauroylsarcosine enriched a 28-kD protein by 25-fold and yielded a vesicle population with high water, but low urea and proton permeabilities. CHIP28k identity was confirmed by NH<sub>2</sub>-terminus sequence analysis. These results indicate that CHIP28k is a major and highly selective water transporting protein in the kidney proximal tubule and thin descending limb of Henle, but not collecting duct.

**T**HERE is a substantial body of functional evidence supporting the existence of facilitated water transporting pathways in multiple segments of the kidney nephron (for review, see Harris et al., 1991; Verkman, 1992). In kidney proximal tubule, water permeability in apical and basolateral plasma membranes is high, weakly temperature dependent, inhibited by mercurial sulfhydryl reagents, and characterized by a high ratio of osmotic-to-diffusional water permeability (Van Heeswijk and Van Os, 1986; Verkman and Wong, 1987; Meyer and Verkman, 1987). Although it is believed that plasma membrane water permeability in proximal tubule is always high to facilitate rapid volume reabsorption driven by small osmotic gradients, various intracellular compartments from proximal tubule cells, including endocytic vesicles (Ye et al., 1989) and clathrin-coated vesicles (Verkman et al., 1989), also contain functional water channels. These vesicles may be involved in the trafficking of water channels to the plasma membranes as part of a constitutive membrane turnover or regulatory process. The thin descending limb of Henle is also highly permeable to water (Imai

et al., 1990); the osmotic extraction of water from thin descending limb in antidiuresis is an important component of the urinary concentrating mechanism, as is the low water permeability of the thin and thick ascending limbs.

The kidney collecting duct also contains water channels (Kuwahara et al., 1991). It is believed that basolateral membrane water permeability in collecting duct principle cells is always high, whereas apical membrane water permeability is regulated by the hormone vasopressin. There is strong morphological (Handler, 1988; Brown, 1989) and functional (Verkman et al., 1988) evidence that vasopressin causes a redistribution of water channels between an intracellular vesicular compartment and the apical plasma membrane by an endocytic-exocytic trafficking mechanism. Water transport across amphibian urinary bladder epithelium is also regulated by vasopressin by a membrane shuttling mechanism (Shi et al., 1990); proteins of molecular size 17, 35, and 55 kD have been identified by SDS-PAGE in purified subcellular vesicles and proposed to be candidate components of the water transporting pathway (Harris et al., 1988; Ver-

bavatz et al., 1989). The relationship between water transporting units in the apical and basolateral membranes of collecting duct and amphibian urinary bladder, and those in proximal tubule and erythrocyte (Macey, 1984) is unknown.

Recent data indicates the existence of a proteinaceous pathway that facilitated water transport. Water permeability in *Xenopus* oocytes expressing mRNA from reticulocyte, kidney, and toad bladder was increased, whereas water permeability in oocytes expressing mRNA from tissues thought not to contain water channels (brain, muscle, and liver) was not different from that in water-injected controls (Zhang et al., 1990; Zhang and Verkman, 1991; Tsai et al., 1991). Radiation inactivation studies gave a ~30-kD target size for the putative water transporting protein in proximal tubule apical membrane vesicles and erythrocytes (Van Hoek et al., 1991; 1992). Recently, a 28-kD erythrocyte protein (CHIP28)<sup>1</sup> with homology to a class of ancient channel-like proteins has been isolated, cloned (Denker et al., 1988; Smith and Agre, 1991; Preston and Agre, 1991) and shown to increase water permeability when expressed in *Xenopus* oocytes (Preston et al., 1992). Although the oocyte expression experiments suggest that CHIP28 is the erythrocyte water channel, the expression of mRNA encoding other membrane transporters, including the glucose transporter (GLUT1, Fischbarg et al., 1990; Zhang et al., 1991) and cystic fibrosis transmembrane regulatory protein when activated by cAMP (CFTR, Hasegawa et al., 1992), also increase oocyte water permeability. Recent measurements of water permeability of liposomes reconstituted with purified CHIP28 protein provide direct evidence that CHIP28 is the erythrocyte water channel (Van Hoek and Verkman, 1992).

The purpose of this study was to define the function and cellular specificity of CHIP28-like proteins in the kidney. Stripped membrane vesicles from rat and human kidney proximal tubule enriched in a 28-kD protein had high water permeability. Liposomes reconstituted with purified protein also had high water permeability that was inhibited by HgCl<sub>2</sub>. cDNA encoding a rat kidney 28-kD protein homologous to CHIP28, referred to as CHIP28k, was cloned and expressed functionally in *Xenopus* oocytes. CHIP28k was shown to be a selective water transporting protein that excluded small ions and urea. The increase in water permeability conferred by injection of oocytes with mRNA from kidney cortex was blocked upon coinjection of an excess of CHIP28k antisense oligonucleotide. Finally, CHIP28k was localized to kidney proximal tubule and thin limb of Henle by in situ hybridization. In a separate antibody localization study (Sabolic et al., 1992), CHIP28k protein was found in plasma and intracellular membranes in proximal tubule and thin descending limb of Henle. The results suggest that the CHIP28k protein is a physiologically important transporter for fluid reabsorption in the proximal nephron. Based on its location and function, inhibition of CHIP28k-mediated water transport by pharmacological blockers should induce a fluid diuresis by overload of distal nephron segments with salt and water, and thus, provide a novel strategy for therapy of refractory edema states.

1. *Abbreviations used in this paper:* BBMV, brush border membrane vesicles; CFTR, cystic fibrosis transmembrane regulatory protein; CHIP28, channel forming integral membrane protein of 28 kD; PCR, polymerase chain reaction.

## Materials and Methods

### mRNA Isolation and Size Fractionation

Fresh superficial cortex and papilla from rat kidney were dissected and frozen in liquid N<sub>2</sub>. Total RNA was isolated by tissue homogenization in guanidinium thiocyanate, followed by phenol extraction and centrifugation through cesium chloride (Zhang et al., 1990).

Poly A<sup>+</sup> RNA was purified twice by affinity chromatography on oligo(dT) cellulose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Size fractionation was performed by centrifugation (45,000 g, 4 h) in a 5–30% sucrose gradient containing 0.2% SDS and 1 mM EDTA. Size-fractionated mRNA was transferred to nitrocellulose and blotted with <sup>32</sup>P-labeled oligo(dT)<sub>30</sub>.

### cDNA Cloning of CHIP28k

Based on the sequence of human erythrocyte CHIP28 (Preston and Agre, 1991), four primers (A–D) were designed with one to two base modifications to contain restriction sites (underlined below) to facilitate cloning the PCR products.

Sense primer A (5' GCC ACC ATG GCC AGC GAG TTC AAG AAG AAG) corresponds to nucleotides -6 ~ +24 of CHIP28.

Antisense primer B (5' GCC GGA TCC CTT CTA TTT GGG CTT CAT CTC) corresponds to nucleotides +793 ~ +822 of CHIP28.

Sense primer C (5' AGC GAG CTG CAG AAG AAG CTC TTC TGG AGG) corresponds to nucleotides +7 ~ +36 of CHIP28.

Antisense primer D (5' CAG CGA AAG CTT CAC GTT GTC CTG GAC CGC) corresponds to nucleotides +133 ~ +162 of CHIP28.

PCR analysis was performed using the GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus, Norwalk, CT). Reactions contained 10 ng of 2.5–4 kb cDNA as template and 25 pmol of primers A and B (1 min at 94°C, 1 min at 45°C, and 2 min at 72°C, 30 cycles). PCR products of the expected size (~800 bp) were purified from a 1.5% agarose gel by the GeneClean kit (Bio 101) and labeled with [ $\alpha$ -<sup>32</sup>P] by T4 DNA polymerase labeling system (Bethesda Research Laboratories, Gaithersburg, MD).

5  $\mu$ g of 2.5–4 kb mRNA from rat kidney cortex was used to construct a directional cDNA library using the SuperScript plasmid system for cDNA synthesis and plasmid cloning (Bethesda Research Laboratories), except that the ds-cDNA was size fractionated by electrophoresis and electroelution. 2.5–4 kb ds-cDNA was ligated into pSPORT1, and transformed into Max Efficiency DH10B competent *Escherichia coli* (Bethesda Research Laboratories). The cDNA library was screened with the <sup>32</sup>P-labeled ~800 bp cDNA probe corresponding to the coding region of CHIP28 (see above). Prehybridization and hybridization were performed at 42°C in 50% formamide with 6  $\times$  SSPE and 0.05  $\times$  Blotto. Filters were washed at high stringency (2  $\times$  SSC, 0.1% SDS four times at 20°C, 1  $\times$  SSC, 0.1% SDS twice at 68°C and 0.2  $\times$  SSC, 0.1% SDS once at 68°C). Positive colonies were further screened by PCR amplification with primers A and B to insure inclusion of the full coding region. Sequence was obtained by the dideoxynucleotide method using a Sequenase kit (version 2.0, US Biochemical Corp., Cleveland, OH). Two additional primers were synthesized to sequence the full coding region.

### In Vitro Translation

To enhance cell free and *Xenopus* oocyte translation efficiency, the CHIP28k coding region was subcloned into plasmid BPI (provided by W. Hansen), which was constructed from plasmid pSP64 containing the 5' region from *Xenopus*  $\alpha$ -globin (Melton et al., 1984; Hasegawa et al., 1992). 20 ng of plasmid DNA containing 2.8 kb of cloned cDNA was used as template to run a 20 cycle PCR amplification with 50 pmol primers A and B (1 min at 94°C, 1 min at 55°C and 2 min at 72°C). This fragment was digested with NcoI and BamHI, gel purified, and ligated into plasmid BPI (which was cut by NcoI and BamHI and dephosphorylated by calf intestinal alkaline phosphatase) to give plasmid PSP CHIP28k. mRNA synthesized by SP6 RNA polymerase was translated into rabbit reticulocyte lysate cell free transcription-linked-translation system in the presence of [<sup>35</sup>S]methionine as described previously (Perara et al., 1986). Translocation of nascent chains into the ER membrane was obtained by addition of crude canine pancreatic microsomes at the start of translation (Walter and Blobel, 1983). After translation at 24°C for 1 h, translation products were digested by addition of proteinase K (final concentration 0.2 mg/ml) for 1 h at 0°C in the presence or absence of 1% Triton X-100. Residual protease was inactivated

by boiling in 10 vol of 1 mM PMSF, 1% SDS, 0.1 M Tris, pH 8.0. Translation products were analyzed by SDS-PAGE and autoradiography.

### ***In Vitro* Transcription/Oocyte Expression**

Plasmid PSP CHIP28k DNA was mini-prepared and purified by PEG8000 precipitation or Magic Miniprep DNA purification system (Promega Corp., Madison, WI). The plasmid was linearized with EcoRI, and transcribed/capped with SP6 RNA polymerase using mCAP mRNA capping kit (Stratagene, La Jolla, CA). Stage V and VI oocytes from *Xenopus laevis* were isolated, defolliculated with collagenase (type 1A, Sigma Chemical Co. [St. Louis, MO], 2 mg/ml, 2 h, 20°C), and stored in Barth's buffer (200 mOsm). Oocytes were microinjected with 50 nl samples of mRNA (0–1 μg/μL) as described (Zhang et al., 1991) and incubated at 18°C for 24–48 h.

Osmotic water permeability in oocytes was measured by a swelling assay (Zhang and Verkman, 1991). The time course of oocyte swelling was measured in response to a 20-fold dilution of the extracellular Barth's buffer with distilled water. Oocyte volume was measured in 1-s intervals by a quantitative imaging method. Temperature control was maintained by a circulating water bath. Oocyte  $P_f$  was calculated from the initial rate of swelling,  $d(V/V_o)/dt$ , by the relation  $P_f = [d(V/V_o)/dt]/[(S/V_o)V_w(Osm_{out}-Osm_{in})]$ , where  $S/V_o = 50 \text{ cm}^{-1}$ ,  $V_w = 18 \text{ cm}^3/\text{mol}$ , and  $Osm_{out}-Osm_{in} = 190 \text{ mOsm}$ .

### ***Two-Electrode Voltage Clamp Measurements***

Membrane currents were measured at specified voltages using a Dagan Instruments TEV-200 voltage clamp apparatus (Dagan Corp., Minneapolis, MN) (Hasegawa et al., 1992). Oocytes (as prepared for water permeability measurements) were impaled with two glass microelectrodes filled with 3 M KCl having resistances of 0.5–1.5 megaohms. Oocytes were superfused continuously with Barth's buffer at room temperature. Current-voltage curves were obtained using a Labmaster analog-to-digital converter and 80386 computer with software written by Hung Pin Kao.

### ***In Situ* Hybridization**

150 base sense and antisense cRNA probes were prepared by PCR amplification of 20 ng CHIP28k cDNA and 50 pmol primers C and D (1 min at 94°C, 1 min at 55°C and 1 min at 72°C, 30 cycles). The amplification product of ~150 bp was cut with PstI and Hind3 and gel purified. Plasmid pSPORT1 was cut by PstI and Hind3, and CIP dephosphorylated before ligation and transformation. 150 base cRNA probes were transcribed by PstI linearization/SP6 RNA polymerase (antisense) and Hind3 linearization/T7 RNA polymerase (sense) with replacement of UTP by [<sup>35</sup>S]UTP in the reaction mixture.

Kidneys of Sprague-Dawley male rats (200–250 gm wt) were fixed in situ with 4% formaldehyde, removed and sliced. After treatment with 30% sucrose, slices were embedded in ornithine carbamyl transferase compound and frozen. 6–12 μm tissue sections were mounted on ionized glass slides for hybridization. Prehybridization and posthybridization steps were performed as described (Pardue, 1988). After treatment with 1 μg/ml proteinase K and 0.25% acetic anhydride, tissue sections were prehybridized for 2 h at 53°C with 50% formamide, 0.3 M NaCl, 20 mM Tris-base (pH 8.0), 5 mM EDTA, 1× Denhardt's solution, 10 mM dithiothreitol and 10% dextran sulfate. Slides were hybridized for 16 h at 55°C in a solution containing the components of the prehybridization solution plus 10 mM dithiothreitol, 2.5 mg/ml tRNA, and the <sup>35</sup>S-labeled cRNA probes (6 × 10<sup>4</sup> cpm/μl). The hybridized slides were dipped in Kodak NTB-2 emulsion at 42°C, diluted 1:1 with distilled water, and exposed for 5 d at 4°C. Development was performed with Kodak D-19 developer (Eastman Kodak Corp., Rochester, NY). Slides were stained with toluidin-blue.

### ***Preparation of Membrane Vesicles and Reconstituted Proteoliposomes***

Proximal tubule apical membrane vesicles (brush border membrane vesicles, BBMV) from homogenates of rat or human renal cortex were separated from basolateral and intracellular membranes by two Mg<sup>2+</sup> precipitation and differential centrifugation steps according to Biber et al. (1981). BBMV were washed twice with 150 mM mannitol, 3 mM EGTA, 20 μg/ml PMSF and 10 mM Tris/HCl (pH 7.8), and snap-frozen dropwise into liquid N<sub>2</sub>. Lipid vesicles were prepared by dissolving 400 mg phosphatidylcholine, 40 mg of phosphatidylinositol and 240 mg cholesterol (all from Sigma Chemical Corp.) (PC:PI:cholesterol = 11:1:11 [mol]) in 1.2 ml diethyl-

ether. The solution was hydrated with 150 mM mannitol, 3 mM EGTA, 10 mM Tris/HCl (pH 7.8) and 0.1 mM DTT at 100 mg/ml lipid. The suspension was vigorously vortexed and evaporated with N<sub>2</sub> to give a gel-like state. The lipids were dispersed by homogenization in 10 ml buffer. The suspension was then diluted to 100 ml and centrifuged overnight at 30,000 rpm in a Beckman 60 Ti rotor (Beckman Instruments, Carlsbad, CA). Pellets were dispersed in 7.2 ml of the above buffer and snap-frozen in liquid N<sub>2</sub>.

Stripped-vesicles were prepared by diluting BBMV (lipid/protein ratio 0.43, w/w) or liposomes to 1 mM phospholipid with 3% N-lauroylsarcosine (100 mM) containing 150 mM mannitol, 3 mM EGTA, 20 μg/ml PMSF, 10 mM Tris/HCl (pH 7.4). The nearly clear suspensions were incubated for 10 min at room temperature and then centrifuged for 1 h at 50,000 rpm in a Beckman Instruments 70.1 Ti rotor. The pellets were washed with buffer (omitting the N-lauroylsarcosine) and stored on ice.

To isolate and reconstitute purified protein, human kidney BBMV were stripped again with 3% N-lauroylsarcosine. The white portion of the pellet was suspended in detergent-free buffer and then solubilized in 250 mM β-octylglucoside and 50 mM CHAPS at 2 mg protein/ml. Solubilized CHIP28k was delipidated by anion exchange chromatography by addition of 1 ml onto an open 0.5 ml Sepharose Q column that was equilibrated previously with 50 mM β-octylglucoside, 0.2 mM EDTA and 10 mM Na phosphate (pH 7.4). Protein yield was 70%. For reconstitution into liposomes, 0.6 mg of the purified protein was mixed with 6 mg lipid (phosphatidylcholine:phosphatidylinositol:cholesterol, 11:1:11). After incubation for 2 h on ice, the mixture was slowly diluted with wash buffer to 20 ml and centrifuged for 2 h at 50,000 rpm (Beckman Instruments 70.1 Ti rotor). The formed proteoliposomes were suspended in 0.2 mM EDTA, 10 mM Na phosphate (pH 7.4) and kept on ice until stopped-flow measurements.

### ***Stopped Flow Assay of Vesicle Water Permeability***

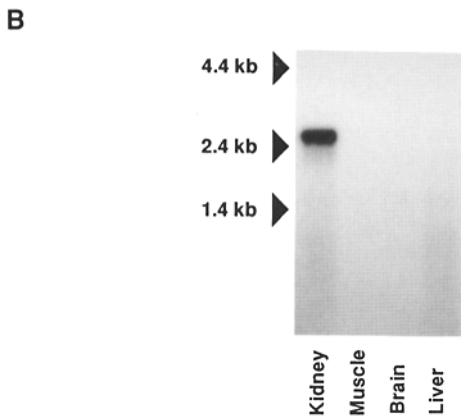
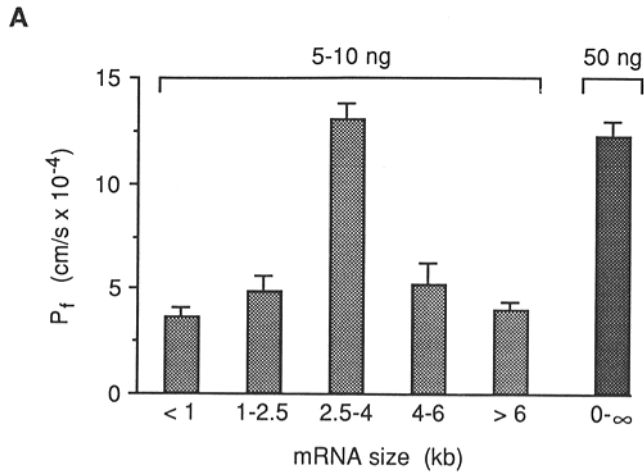
Membranes were diluted in 150 mM mannitol, 2 mM MgCl<sub>2</sub> and 10 mM Tris/HCl (pH 7.4) and mixed in a Hi-Tech SF51 stopped-flow apparatus with an equal volume of buffer containing 400 mM (instead of 150 mM) mannitol to give an inwardly-directed 125 mOsm gradient. Scattered light intensity at 90° was measured using three different time scales in sequence to follow rapid and slow osmotic responses in every measurement. In some experiments, membranes were incubated with 2% DMSO (Van Hoek et al., 1990) or 100 μM HgCl<sub>2</sub> for 5 min before stopped flow measurement.  $P_f$  (cm/s) was measured from the time course of light scattering and vesicle diameter as described previously (Meyer and Verkman, 1987). Experiments were carried out at 10°C to minimize water permeability across the lipid membrane.

## **Results**

### ***cDNA Cloning and Translation of the Kidney Water Transporter***

A size-fractionated cDNA library from rat kidney cortex was constructed for isolation of cDNA homologous to erythrocyte CHIP28. Fig. 1 A shows the osmotic water permeability of *Xenopus* oocytes microinjected with size-fractionated and total mRNA from rat kidney cortex. Water permeability in oocytes injected with 5–10 ng of 2.5–4 kb mRNA was similar to that in oocytes injected with 50 ng of unfractionated mRNA. The increase in water permeability was inhibited by >80% by 0.3 mM HgCl<sub>2</sub> (not shown), similar to the mercurial sensitivity measured in the original kidney membranes (Meyer and Verkman, 1987). These results suggest that a single mRNA in the 2.5–4-kb size range encodes a water channel in kidney cortex. Fig. 1 B shows a Northern blot in which mRNA from kidney, but not brain, liver and muscle, hybridized to a probe homologous to CHIP28. The transcript size was ~2.8 kb.

Based on the cDNA sequence flanking the 807 bp coding region of CHIP28 (Preston and Agre, 1991), oligonucleotide primers A and B were constructed to amplify an ~800 bp



**Figure 1.** Size of mRNA encoding the kidney water channel. (A) Osmotic water permeability ( $P_f$ ) in *Xenopus* oocytes injected with 5–10 ng of (poly A+) mRNA from rat kidney cortex of the indicated size or 50 ng of unfractionated mRNA.  $P_f$  was measured at 10°C from the time course of oocyte swelling in response to a 20-fold dilution of the extracellular osmolality. Measurements were performed 48 h after injection. Error bars are the SE for 8–10 oocytes in one set of measurements typical of three. (B) Northern blot of mRNA (1–2  $\mu$ g/lane) from rat kidney, liver, brain, and muscle probed with a  $^{32}$ P-labeled 800 bp DNA probe corresponding to the CHIP28k coding region. Prehybridization was performed with salmon sperm DNA at 42°C for 2 h and hybridization with the DNA probe at 42°C for 16 h.  $\beta$ -actin controls showed similar hybridization in each lane.

DNA fragment from rat kidney cortex cDNA. The ~800-bp probe was used to screen a cDNA library prepared from 2.5–4 kb rat kidney cortex mRNA. Positive colonies were screened for the full-length coding region by PCR amplification. The insert size of the largest positive colonies was ~2.8 kb, in agreement with that determined by Northern hybridization in Fig. 1 B. Interestingly, ~0.2% of bacterial colonies from the directional size-fractionated cDNA library contained the full-length coding sequence, indicating that mRNA encoding the kidney homolog of CHIP28 (referred

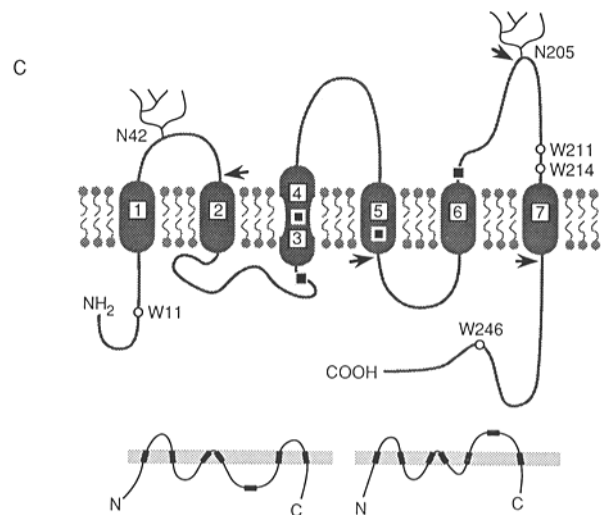
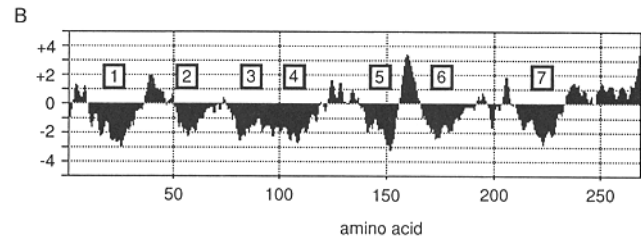
**Figure 2.** Sequence and proposed structure of CHIP28k. (A) cDNA and amino acid sequence of the CHIP28k coding region. Underline indicates that the nucleotide or amino acid of CHIP28k is different from that of human erythrocyte CHIP28. (B) Hydrophilicity anal-

cDNA sequence of CHIP28k coding region

```

1  ATG  GCC  AGC  GAG  TTC  AAG  AAG  AAG  CTC  TTC  TGG  AGG  GCI  GTG  GTG  GCI  GAG  TTC
   M   A   S   E   F   K   K   K   L   F   W   R   A   V   V   A   E   F
55  CTG  GCC  AIG  ACC  CTC  TTC  GTC  TTC  ATC  AGC  ATC  GGT  TCT  GCC  CTA  GGC  TTC  AAI
   L   A   M   T   L   F   V   F   I   S   I   G   S   A   L   G   F   N
109 TAC  CCA  CTG  GAG  AGA  AAC  CAG  ACG  CIG  GTC  CAG  GAC  AAI  GTG  AAG  GTG  TCA  CTG
   Y   P   L   E   R   N   Q   T   L   V   Q   D   N   V   K   V   S   L
163  GCC  TTI  GGI  CTG  AGC  ATC  GCI  ACI  CTG  GCC  CAA  AGT  GTG  GGI  CAC  ATC  AGI  GGI
   A   F   G   L   S   I   A   T   L   A   Q   S   V   G   H   I   S   G
217  GCI  CAC  CTC  AAC  CCA  GCG  GTC  ACA  CTG  GGG  CTI  CTG  CTC  AGC  TGI  CAG  ATC  AGC
   A   H   L   N   P   A   V   T   L   G   L   L   L   S   C   Q   I   S
271  ATC  CTC  CGG  GCI  GTC  ATG  TAI  ATC  ATC  GCC  CAG  TGI  GTG  GGA  GCC  ATC  GTI  GCC
   I   L   R   A   V   M   Y   I   I   A   Q   C   V   G   A   I   V   A
325  ICC  GCC  ATC  CTC  TCC  GGC  ATC  ACC  TCC  TCC  CTG  GAG  AAC  TCA  CTT  GGC  CGA
   S   A   I   L   S   G   I   T   S   S   L   L   E   N   S   L   G   R
379  AAT  GAC  CTG  GCT  CGA  GGT  GTG  AAC  TCC  GGC  CAG  GGC  CTG  GGC  ATI  GAG  ATC  ATI
   N   D   L   A   B   G   V   N   S   T   G   C   G   C   T   G   A   I   I
433  GGC  ACC  CTG  CAG  CTG  GTG  CTG  TGC  GTI  CTG  GCT  ACC  ACI  GAC  CGG  AGG  CGC  CGA
   G   T   L   Q   L   V   L   C   V   L   A   T   T   D   R   R   R
487  GAC  ITA  LGG  GGC  TCA  GCC  CCA  CTT  GCC  ATI  GGC  ITG  TCT  GTG  GCI  CTT  GGA  CAC
   D   L   G   G   S   A   P   L   A   I   G   L   S   V   A   I   V   H
541  CTG  CTG  GCC  ATT  GAC  TAC  ACT  GGC  TGT  GGG  ATC  AAC  CCT  GCC  CGG  TCA  TTT  GGC
   L   L   A   I   D   Y   T   G   C   I   N   P   A   R   S   F   F   G
595  TCI  GCI  GTG  CTC  ACC  CCG  AAC  TTC  ICA  AAC  CAC  TGG  ATT  TTC  TGG  GTG  GGA  CCA
   S   A   V   L   T   B   N   F   S   N   H   W   I   F   W   V   G   P
649  TTC  ATI  GGG  AGI  GCC  CTG  GCA  GTG  CTG  ATC  TAI  GAC  TTC  ATC  CTG  GCC  CCA  CGC
   F   I   G   S   A   L   A   V   L   I   Y   D   F   I   R   A   P   R
703  AGC  AGC  GAC  ITI  ACA  GAC  CGC  ATG  AAG  GTG  TGG  ACC  AGI  GGC  CAG  GTG  GAG  GAG
   S   S   D   E   T   D   R   M   K   V   W   T   S   G   Q   V   E   E
757  TAT  GAC  CTG  GAT  GCI  GAI  GAI  ATC  AAC  TCC  AGG  GTG  GAG  ATG  AAG  CCC  AAA  TAG
   Y   D   L   D   A   D   D   I   N   S   R   V   E   M   K   P   K

```



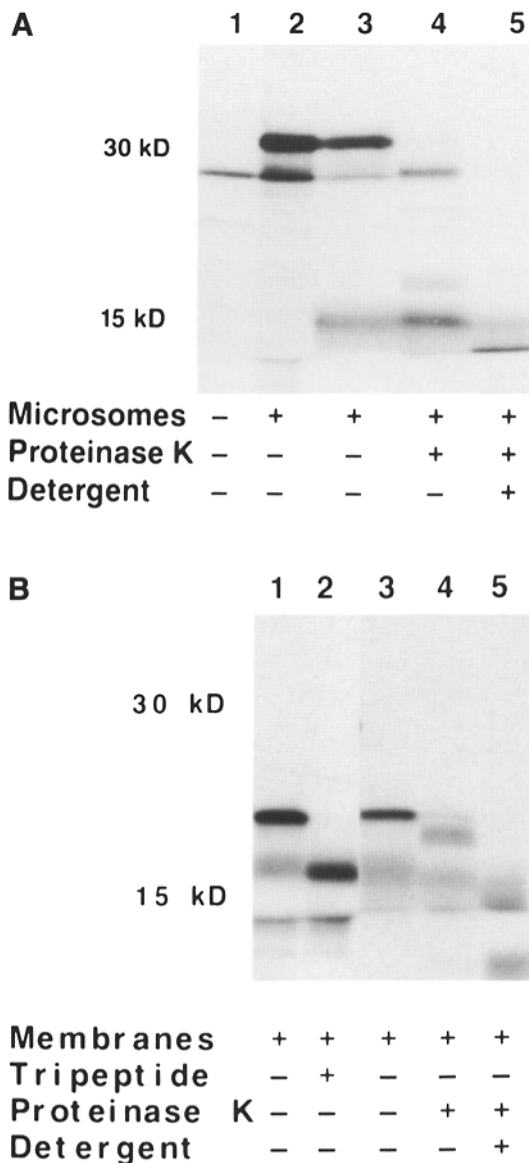
ysis of CHIP28k by Kyte-Doolittle method showing seven segments of sufficient length to span the membrane. (C) Putative membrane topology of CHIP28k assuming a series of transmembrane  $\alpha$ -helical domains (see text for details). The potential glycosylation sites (N), tryptophans (W), cysteines (box), and potential phosphor-tyrosylation sites (arrow) are shown. The second and third possible transmembrane domains are depicted together. Two alternative topologies in which one of the hydrophobic domains is in an extra-membrane location are provided (see text).

to as CHIP28k) was very prevalent. Estimating that the 2.5–4 kb mRNA made up ~10% of total mRNA based on a poly(dT)<sub>30</sub>-probed Northern blot, it was estimated that ~1 out of 5,000 mRNAs from kidney cortex encoded CHIP28k.

The cDNA sequence of the CHIP28k coding region (Fig. 2 A) showed 88% identity to erythrocyte CHIP28; the amino acid sequence showed 94% identity to CHIP28. The bases and amino acids that differed from those of CHIP28 are underlined. The open reading frame was 807 bp encoding a protein with 269 amino acids of predicted molecular size of 28.8 kD. Hydrophilicity analysis (Kyte-Doolittle, Fig. 2 B) indicates that the predicted protein is very hydrophobic, containing five hydrophobic domains of ~20 amino acids, one hydrophobic domain of ~40 amino acids, and short hydrophilic domains. Assuming that CHIP28k is a multi-spanning transmembrane protein with intramembrane  $\alpha$ -helical structure, a proposed topology is shown in Fig. 2 C. Assuming that the NH<sub>2</sub>-terminus is cytoplasmic and that the third hydrophobic sequence gives only a single membrane crossing, there are two potential N-linked glycosylation sites at residues Asn42 and Asn205. There are four tryptophan (*W*) residues for mapping studies by fluorescence quenching and energy transfer, four possible cysteine target sites (*dark box*) for mercurial inhibition, and four potential phosphorylation sites (*arrows*). Although there is good evidence that the NH<sub>2</sub> and COOH-termini are cytoplasmic (Preston and Agre, 1991; Skach, Shi and Verkman, unpublished results), the topology of the rest of the protein is not yet established. Two possible alternative topologies are shown at the bottom of Fig. 2 C in which one of the hydrophobic sequences resides outside of the membrane.

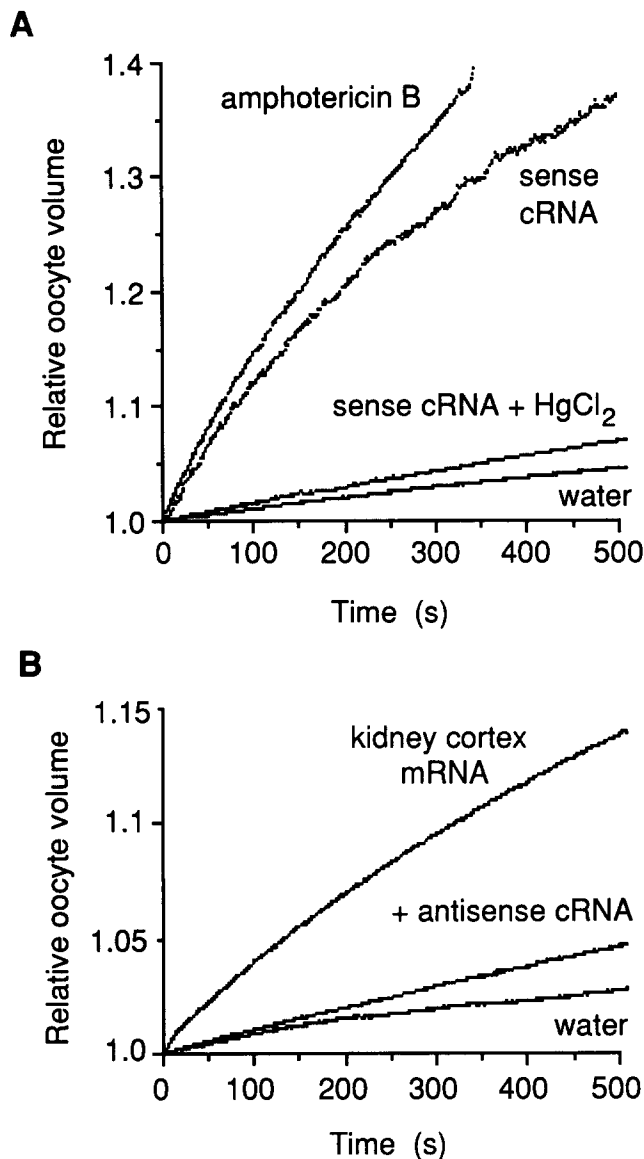
Fig. 3 A shows SDS-PAGE of in vitro translated proteins. When translated in the rabbit reticulocyte cell-free translation system, clone PSP CHIP28k generated a single polypeptide chain which migrated at the expected size of 28 kD. When canine pancreatic microsomes were added during translation, nascent chains underwent translocation into the microsome lumen and were glycosylated as shown by a shift in migration (lanes 2 and 3). Glycosylation was confirmed by showing that addition of a tripeptide inhibitor of N-linked glycosylation (AcAsn-Tyr-Thr) during translation prevented this shift (data not shown). Digestion by proteinase K (lane 4) generated several small protected fragments, demonstrating that the 28-kD protein spans the bilayer, and that a portion or portions of the chain remain extraluminal and accessible to protease. These fragments were digested completely when microsome integrity was abolished by a nondenaturing detergent (lane 5), confirming that protection was due to luminal translocation rather than intrinsic protease sensitivity. The presence of several distinct small protected fragments suggests that the protein has multiple membrane spanning regions and hence several cytosolic domains which are accessible to protease.

PSP CHIP28k was then truncated by PstI at bp 443 and translated in vitro. In the presence of microsomal membranes, a ~19-kD protein was generated that shifted to ~16 kD in the presence of the tripeptide inhibitor of N-linked glycosylation (Fig. 3 B, lanes 1 and 2). When released from ribosomes by addition of puromycin (1 mM), a portion of this glycosylated chain remained resistant to digestion by proteinase K in the absence (lane 4), but not in the presence (lane 5) of detergents. Because Asn42 is the only potential



**Figure 3.** In vitro translation of CHIP28k cDNA. (A) Clone PSP CHIP28k was translated in rabbit reticulocyte lysate in the absence (lane 1) or presence (lanes 2–5) of canine pancreatic (ER-derived) microsomal membranes. 1  $\mu$ l of total translation products were analyzed by SDS-PAGE and autoradiography. Alternatively, 5  $\mu$ l of a 1:10 dilution of translation products were analyzed following mock digestion (lane 3), proteinase K digestion (lane 4), or proteinase K digestion in the presence of nondenaturing detergent (lane 5). (B) After digestion of PSP CHIP28k with PstI, the truncated cDNA was translated as in A in the absence (lanes 1, 3–5) or presence (lane 2) of a tripeptide inhibitor of N-linked glycosylation (AcAsn-Tyr-Thr). Proteolysis of translation products (lanes 3–5) was carried out as in A.

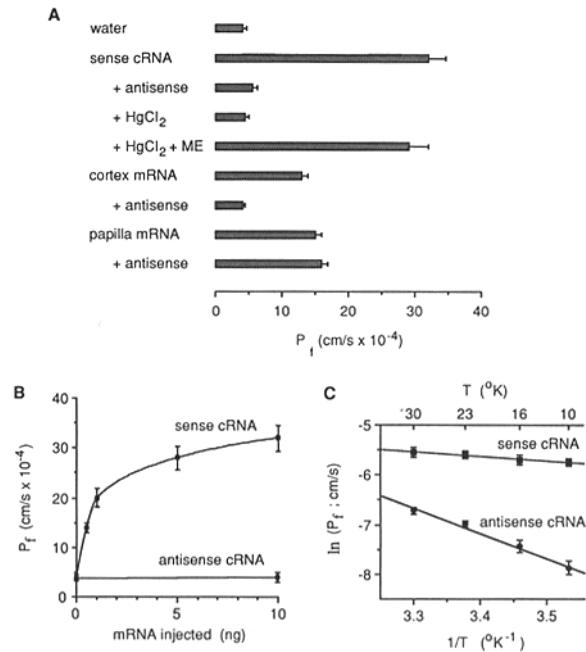
N-linked glycosylation site in this truncated polypeptide, the region of CHIP28k following the first putative transmembrane  $\alpha$ -helix must undergo translocation into the ER lumen. If the hydrophobic domain preceding Asn42 spans the membrane as predicted, then the amino terminus of CHIP28k resides in the cytoplasm as diagrammed in Fig. 2 C. Further, because full length CHIP28k appears to undergo a single glycosylation event (~3 kD shift in size), Asn205 is probably not glycosylated.



**Figure 4.** Expression of CHIP28k and kidney mRNA in *Xenopus* oocytes. (A) Oocytes were injected with 50  $\mu$ l of water or a 0.2  $\mu$ g/ $\mu$ l solution of in vitro transcribed CHIP28k mRNA (sense mRNA). Osmotic water permeability was assayed at 10°C after a 24–30 h incubation at 18°C. Where indicated, the water-injected oocyte was incubated with 300  $\mu$ g/ml amphotericin B for 5 min before measurement; the mRNA-injected oocyte was assayed in hypoosmotic buffer containing 0.3 mM HgCl<sub>2</sub>. (B) Oocytes were injected with 50 ng mRNA from kidney cortex. Where indicated, 10 ng of a 150 bp antisense RNA was coinjected with the cortex mRNA.

#### Expression of CHIP28k in *Xenopus* Oocytes

Fig. 4 shows the time course of oocyte swelling in response to an osmotic gradient (inside 200 mOsm, outside 10 mOsm). Oocyte volume was determined in 1-s intervals by real-time image analysis. Fig. 4 A shows that microinjection of oocytes with in vitro transcribed sense mRNA encoding CHIP28k strongly increased osmotic water permeability to a value similar to that achieved by incubation of oocytes with high concentrations of the pore-forming agent amphotericin B. The increase in oocyte water permeability conferred by CHIP28k mRNA was inhibited by HgCl<sub>2</sub>. Fig. 4 B shows

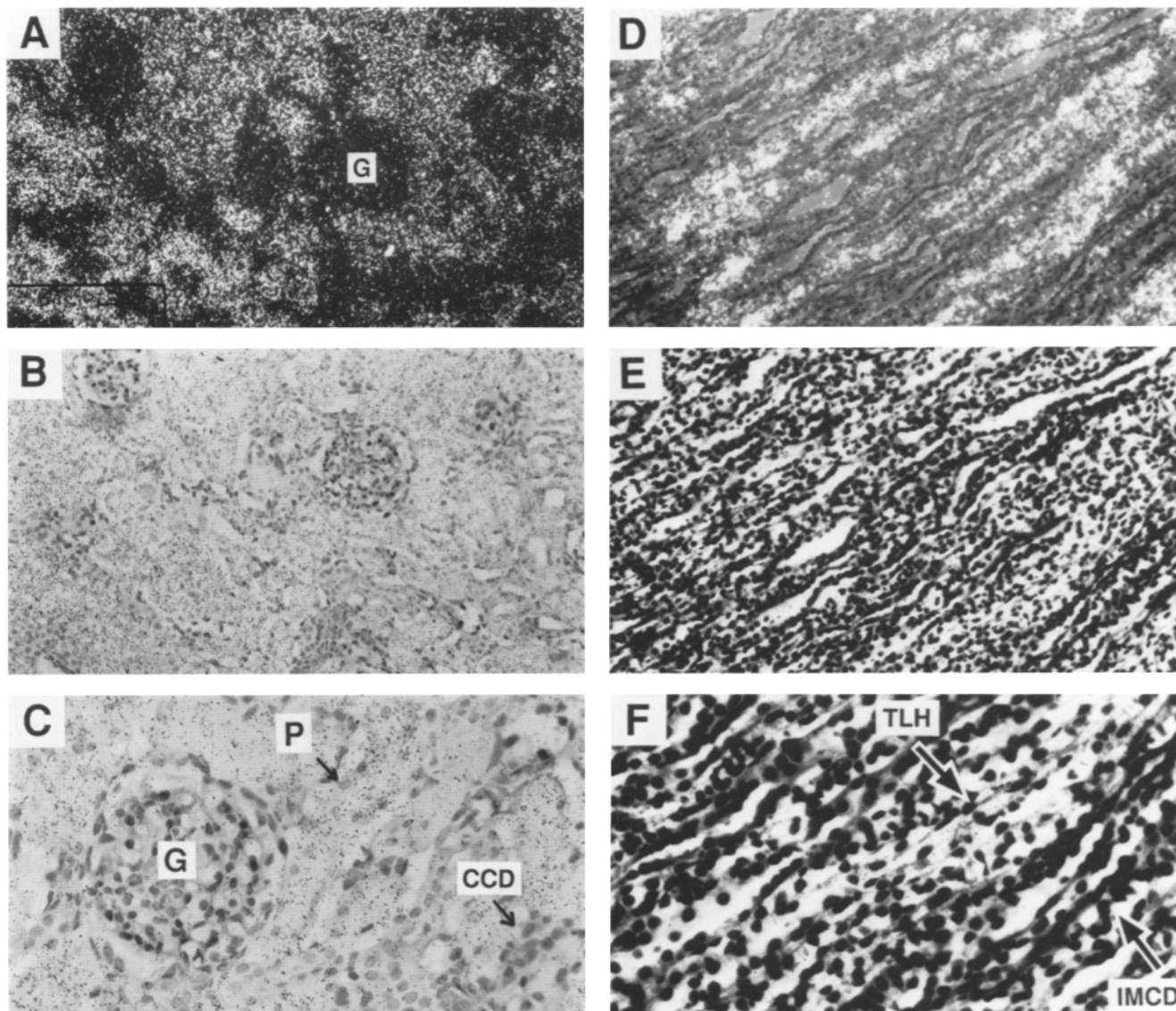


**Figure 5.** Osmotic water permeability of oocytes expressing CHIP28k. (A) Summary of oocyte water permeabilities (mean  $\pm$  SE, 12–16 oocytes) measured at 10°C at 24–30 h after microinjection. Procedures were as described in the legend to Fig. 4. Where indicated (+HgCl<sub>2</sub> +ME), 5 mM  $\beta$ -mercaptoethanol was added after the 5 min incubation with 0.3 mM HgCl<sub>2</sub>. Amounts of mRNA were 10 ng (sense CHIP28k) and 50 ng (kidney cortex and papilla); the amount of antisense CHIP28k cRNA was 10 ng. (B) Dependence of oocyte water permeability on the amount of injected CHIP28k cRNA. (C) Arrhenius plot of the temperature dependence of  $P_f$  in oocytes injected with 10 ng of sense or antisense CHIP28k cRNA. Fitted slopes gave activation energies of  $2.1 \pm 0.6$  kcal/mol (sense) and  $11 \pm 2$  kcal/mol (antisense).

that the increase in oocyte water permeability conferred by expression of mRNA from kidney cortex was blocked by coinjection of an excess of a 150 base CHIP28k antisense RNA. Therefore, the increase in oocyte water permeability is due to expression of mRNA encoding CHIP28k, and/or mRNAs homologous to CHIP28k.

The results of a series of oocyte water permeability measurements are given in Fig. 5 A. Experiments were performed at 10°C to minimize endogenous (channel independent) oocyte water permeability. The high water permeability conferred by the CHIP28k sense RNA was blocked by coinjection with an excess of CHIP28k antisense RNA, or by addition of HgCl<sub>2</sub> at the time of water transport assay. Addition of  $\beta$ -mercaptoethanol after HgCl<sub>2</sub> reversed the inhibition, indicating that the HgCl<sub>2</sub> inhibition is not a nonspecific toxic effect. The increase in oocyte water permeability conferred by mRNA from renal cortex, but not renal papilla, was blocked by CHIP28k antisense RNA. This result suggests that the (vasopressin sensitive) water channel encoded by mRNA from renal papilla is not CHIP28k.

Fig. 5 B shows the relationship between oocyte water permeability and the amount of injected sense CHIP28k mRNA. Oocyte water permeability increased by >twofold with injection and expression of 0.5 ng CHIP28k mRNA. There was no effect of injection of 10 ng of a full length antisense CHIP28k mRNA on oocyte water permeability. Fig.



**Figure 6.** In situ hybridization of rat kidney cortex (*left*) and papilla (*right*) probed by CHIP28k antisense cRNA. Photographs shown are darkfield (*A* and *D*), and brightfield stained by Toluidin blue at low (*B* and *E*) and high (*C* and *F*) magnification. Areas of hybridization in *A* and *D* are seen as bright dots and in *B*, *C*, *E*, and *F* as dark granules. *G*, glomerulus; *P*, proximal tubule; *CCD*, cortical collecting duct; *IMCD*, inner medullary collecting duct; *TLH*, thin limb of Henle.

5 *C* shows the temperature dependence of oocyte water permeability in the form of an Arrhenius plot. The activation energies ( $E_a$ ), given from the slope of the fitted lines, were  $2.1 \pm 0.6$  kcal/mol (sense) and  $11 \pm 2$  kcal/mol (antisense). The  $E_a$  for antisense cRNA was similar to that of 10 kcal/mol reported previously for water-injected oocytes which do not contain water channels. The  $E_a$  for sense cRNA was low, indicating the presence of functional water channels. Taken together, the high water permeability, low  $E_a$ , and  $HgCl_2$  inhibition support the conclusion that CHIP28k is a kidney water channel.

To determine whether CHIP28k conducts ions as well as water, two-electrode voltage clamp measurements were carried out on oocytes injected with water or 10 ng CHIP28k mRNA. Current-voltage relationships were measured at holding potentials between  $-80$  and  $+20$  mV. In five water and mRNA-injected oocytes, the average (mean  $\pm$  SE) whole oocyte conductances and reversal potentials were  $1.6$

$\pm 0.5$   $\mu S$  and  $-43 \pm 4$  mV (water-injected) and  $1.7 \pm 0.4$   $\mu S$  and  $-38 \pm 8$  mV (mRNA-injected), respectively. In control measurements, oocytes injected with 10 ng of RNA encoding the cystic fibrosis gene product CFTR had reversal potentials of  $-25$  to  $-35$  mV and conductances of  $70$ – $120$   $\mu S$  after stimulation by cAMP agonists (Hasegawa et al., 1992). These studies indicate that CHIP28k is a selective water transporter and are in agreement with measurements in oocytes expressing CHIP28 reported by Preston et al. (1992).

#### **Cellular Localization of CHIP28k mRNA by In Situ Hybridization**

150 base sense and antisense  $^{35}S$ -labeled cRNA oligonucleotides were used to probe thin sections from rat kidney. Fig. 6 *A* shows a darkfield micrograph of kidney cortex where the areas of hybridization are visualized as bright dots. Fig. 6 *B*

shows the same field by brightfield microscopy after staining with Toluidin blue, showing strong hybridization (*dark granules*) to proximal tubule, and little if any hybridization to glomerulus and cortical collecting duct. Fig. 6 C shows a brightfield stained photograph taken at higher magnification in which the areas of hybridization are seen as dark granules. For all sections, the sense cRNA control showed no specific hybridization (not shown).

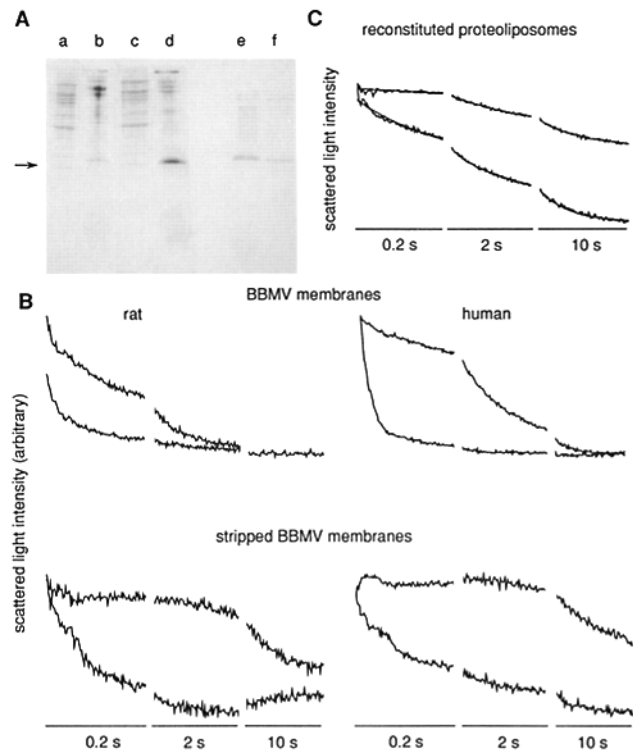
Fig. 6 D is a darkfield section from rat kidney papilla showing hybridization to a subpopulation of tubules. Fig. 6 E is a brightfield, stained section, showing uptake in thin limbs of Henle with no hybridization in inner medullary collecting duct. Fig. 6 F is a brightfield stained photograph taken at higher magnification. These results indicate that mRNA encoding CHIP28k localizes to epithelial cells lining the kidney proximal tubule and thin limb of Henle. Although the descending and ascending limbs cannot be distinguished here, separate antibody binding studies show CHIP28k immunolocalization to descending, but not ascending limbs (Sabolic et al., 1992). The lack of hybridization to collecting duct is consistent with the finding above that antisense CHIP28k RNA does not block the increase in water permeability in oocytes expressing mRNA from kidney papilla.

### Water Transport in Apical Membrane Vesicles and Reconstituted Proteoliposomes

Experiments were carried out to determine whether CHIP28k was detectable and functional as a water channel in brush border membrane vesicles derived from proximal tubule apical membrane. Fig. 7 A shows that the original BBMVs contained multiple membrane proteins (lanes a [rat] and c [human]). Stripping of vesicles with N-lauroylsarcosine resulted in >25-fold enrichment of a stained band at 28 kD (lanes b and d) with an increase in vesicle lipid-to-protein ratio (Table I). To prove that the stained band at 28 kD (lane d) was CHIP28k, NH<sub>2</sub>-terminus sequence analysis was performed by blotting on a polyvinylidene difluoride membrane and Edman degradation. The derived amino acid sequence (NH<sub>2</sub>-[M]-A-S-E-F-K-K) corresponded to that predicted by the CHIP28k cDNA sequence. No other proteins comigrated in the 28-kD region. The NH<sub>2</sub>-terminus was not blocked. Lane f shows the purified CHIP28k protein after anion exchange chromatography.

Figs. 7, B and C show that the vesicles and reconstituted proteoliposomes were highly water permeable with a rapid volume decrease in response to an osmotic gradient; osmotic water transport was inhibited strongly by HgCl<sub>2</sub> or DMSO. Average P<sub>f</sub> values are given in Table I. Native BBMVs had a high P<sub>f</sub> that was strongly inhibited by DMSO and HgCl<sub>2</sub>. The high P<sub>f</sub> and inhibitory potency were maintained after stripping with N-lauroylsarcosine. In control studies, protein-free liposomes had low water permeability that was not altered by stripping with N-lauroylsarcosine or by addition of inhibitors. Proteoliposomes reconstituted with relatively small amounts of purified protein (lipid-to-protein ratio ~330) had a >fivefold increase in P<sub>f</sub> compared to protein-free liposomes; the incremental P<sub>f</sub> associated with CHIP28k was inhibited by HgCl<sub>2</sub> and DMSO.

To determine the transport specificity of the water transporter, the permeability of stripped BBMVs to urea and protons was measured. Urea permeability was measured from the time course of light scattering in response to a 500 mM inwardly directed gradient of urea (Meyer and Verkman,



**Figure 7.** SDS-PAGE and water permeability of proximal tubule apical vesicles and reconstituted proteoliposomes. (A) 12% SDS-polyacrylamide gel of proximal tubule apical membrane vesicle preparations. The gel was fixed with methanol/acetic acid and stained with Coomassie blue in acetic acid. Lanes consist of: rat BBMVs before (a) and after (b) stripping with N-lauroylsarcosine, human BBMVs before (c) and after stripping once (d) and twice (e). Lane f shows the purified protein after delipidation and anion exchange chromatography. The arrow indicates 28 kD. (B and C) Time course of scattered light intensity in rat and human BBMVs, before and after stripping with N-lauroylsarcosine (B), and proteoliposomes reconstituted with purified human CHIP28k (C). Vesicles were subjected to a 125 mM inwardly-directed mannitol gradient at 10°C. Relative vesicle volume was determined from scattered light intensity. The data are shown on three different time scales in sequence to follow the full time course of volume decrease. The upper curve in each panel indicates the presence of 2% DMSO.

1987). Urea permeability was in the range  $0.8\text{--}1.5 \times 10^{-6}$  cm/s for rat and human stripped-BBMVs, and not inhibited by 0.2 mM phloretin. Passive proton permeability was measured from the kinetics of acidification (pH<sub>in</sub> 7.6, pH<sub>out</sub> 6.5) of stripped-BBMVs loaded with carboxyfluorescein in the presence of 100 mM KCl (intravesicular and external) and the ionophore valinomycin (Verkman, 1987). Proton permeability (P<sub>H</sub>) was relatively low (0.005–0.01 cm/s) and not inhibited by 0.1 mM HgCl<sub>2</sub>. Taken together, these results suggest that CHIP28k is a selective water transporting protein that excludes protons, ions and small solutes.

### Discussion

Several lines of evidence suggest that the CHIP28k protein is an important water transporter in the proximal nephron. mRNA encoding CHIP28k was very abundant in renal cortex, similar to the abundance of mRNA encoding band 3 (AE1) in erythroid cells. In vitro transcribed mRNA encod-



**Table I. Osmotic Water Permeability in Apical Membrane Vesicles and Reconstituted Proteoliposomes from Proximal Tubule**

	protein	lipid	lipid/protein	D	control	+DMSO	+Hg <sup>2+</sup>
	mg	mg	mol:mol	nm		$P_f(\text{cm/s} \times 0.01)$	
Rat BBMV	12	5.2	0.43	285	2.8	0.15	0.15
Human BBMV	18	7.4	0.41	240	4.0	0.07	0.10
Stripped rat BBMV	0.48	1.5	2.5	165	1.4	0.01	0.04
Stripped human BBMV	0.76	2.1	2.7	160	2.4	0.01	0.03
Liposomes	—	10	—	700	0.03	0.03	0.03
Stripped liposomes	—	10	—	400	0.03	0.03	0.03
Reconstituted liposomes	0.02	6	330	365	0.16	0.03	0.04

Data are shown for the original and N-lauroylsarcosine stripped apical membrane vesicles from rat and human, and for proteoliposomes reconstituted with highly purified human kidney CHIP28 (see Methods for details). Protein was determined by Lowry assay and total phospholipid by phosphorus assay. Average vesicle diameter (D) was determined by Coulter counting. Osmotic water permeability ( $P_f$ ) was measured by stopped flow light scattering (e.g., see Fig. 7) in the absence or presence of the inhibitors DMSO or HgCl<sub>2</sub>.

ing CHIP28k strongly increased water permeability in *Xenopus* oocytes; the HgCl<sub>2</sub> sensitivity and weak temperature dependence of the expressed water transporter was similar to that in the original tissue. The ability of an antisense CHIP28k RNA to block the increase in water permeability by mRNA from kidney cortex suggests that CHIP28k (or homologous proteins) is the major water transporter in kidney cortex. It is not possible, however, to rule out the existence of other water transporting proteins that are not expressed in oocytes. In particular, the antisense CHIP28k RNA did not block functional expression of water transporter(s) encoded by mRNA from kidney papilla. The experiments carried out on apical membrane vesicles from proximal tubule enriched in CHIP28k indicate that CHIP28k is a very abundant water transporting protein. The CHIP28k in situ hybridization pattern indicates localization in epithelial cells of the kidney proximal tubule and thin descending limb of Henle, but not in glomerulus, vascular cells, and collecting duct. Recently, a polyclonal rabbit antibody raised against purified human erythrocyte CHIP28 was shown to recognize CHIP28k on Western blots of apical membrane vesicles, basolateral membrane vesicles, and endosomes from rat kidney cortex (Sabolic et al., 1992). Immunocytochemistry by light and electron microscopy indicated localization of CHIP28k protein to plasma membranes and endosomes in proximal tubule and thin descending limb of Henle. Interestingly, there was weak staining of plasma membranes in principal cells in cortical, but not medullary collecting duct. In situ hybridization studies of nonrenal tissues indicated that CHIP28k has a wide and highly selective distribution in alveolar and colonic crypt epithelium, corneal endothelium, choroid plexus, red splenic pulp and others (Hasegawa et al., 1993).

The amino acid sequence and hydropathy analysis of CHIP28k suggests at least six membrane spanning domains with relatively small connecting loops. CHIP28k and CHIP28 are homologous to an ancient class of channel proteins which include MIP (major intrinsic protein of the lens), GlpF (glycerol facilitator protein in *E. coli*), BiB (*Drosophila* big brain protein) and several plant proteins (Preston and Agre, 1991). Although some of these proteins are ion transporters, their permeability to water has not been measured. The short extra-membrane domains in CHIP28k are consistent with the finding that the proximal tubule water transporter is difficult to inhibit by proteases at the cytoplasmic or extracytoplasmic surfaces (Sabolic et al., 1992). There are four cysteines in CHIP28k, two of which are predicted

to reside in extra-membrane segments and thus, may be involved in HgCl<sub>2</sub> inhibition. Recent site-directed mutagenesis experiments indicate that cysteine 189 is the site of action of mercurials (Zhang, Van Hoek and Verkman, unpublished results). The cell-free translation data indicate N-linked glycosylation of extracytoplasmic residue Asn42; assuming  $\alpha$ -helical transmembrane domains, it is likely therefore that the NH<sub>2</sub>-terminus of CHIP28k resides in the cytoplasm. Selective digestion data of CHIP28 in erythrocytes and inside-out ghost membrane suggested that the COOH terminus is also cytoplasmically oriented (Smith and Agre, 1991). Recent spectroscopic studies of CHIP28 secondary structure by circular dichroism and Fourier transform infrared spectroscopy support the conclusion that CHIP28 contains multiple membrane-spanning  $\alpha$ -helical domains (Van Hoek et al., 1993).

The structure of the functional water transporting unit cannot be determined at this time. Radiation inactivation studies showing a single ~30-kD functional target size suggest that the CHIP28k monomer is the functional water transporting unit (Van Hoek et al., 1991; 1992). If the functional unit were a tightly assembled dimer or tetramer, then the target sizes would have been ~60 or ~120 kD, respectively. Further, if the functional water transporting unit were a transiently-assembled oligomer, then the target analysis experiments would have revealed a nonlinear (concave downward) relationship between  $\ln(P_f)$  and radiation dose (Verkman et al., 1984). The hydropathy analysis of CHIP28k is consistent with the assignment of a monomeric functional unit; however, the possibility of water passage between subunits of a multimeric assembly of CHIP28k monomers cannot be ruled out, nor can an assembly of CHIP28k proteins with membrane lipids. However, the high water permeability in proteoliposomes reconstituted with CHIP28 (Van Hoek and Verkman, 1992) rules out a heteromultimeric assembly of CHIP28k and other membrane proteins. Based on studies of sedimentation coefficients and cross-linking in glutaraldehyde, Smith and Agre (1991) concluded that the erythrocyte CHIP28 protein exists as a multisubunit oligomer. Recent freeze-fracture electron micrographs with rotary shadowing of proteoliposomes reconstituted with CHIP28 suggested a tetrameric assembly in the membrane (Verbavatz et al., 1992). In these morphological studies, there was a fairly uniform population of intramembrane particles with 10-nm diam, each consisting of a symmetrical arrangement of four smaller units arranged around a central depression. Further

analyses of protein cross-linking and selected CHIP28k mutations will be required to determine whether multimeric assembly of CHIP28k is required for water transport function.

The single channel water permeability ( $p_f$ , in  $\text{cm}^3/\text{s}$ ) for CHIP28 was estimated from reconstitution data to be  $6.8 \times 10^{-14} \text{ cm}^3/\text{s}$  (Van Hoek and Verkman, 1992), assuming that the functional subunit is a monomer. Assuming similar  $p_f$  for CHIP28k, the amount of CHIP28k in the proximal tubule apical membrane can be estimated. Given a measured  $P_f$  of  $0.07 \text{ cm/s}$  ( $37^\circ\text{C}$ ), there are  $\sim 7,000$  CHIP28k molecules per  $\mu^2$  membrane area. If the membrane is 50% protein and 50% lipid, then CHIP28k makes up 5–6% of total membrane protein. This calculation is consistent with the high measured abundance of CHIP28k mRNA and protein and indicates that many water channels are required to increase membrane water permeability only  $\sim 50$ -fold over that through lipid bilayers in the absence of water channels. Further, assuming that the water channel is a narrow water-filled pore, the pore radius,  $r_o$ , can be estimated by the equation,  $r_o^2 = p_f L / \pi D_w$ , where  $L$  is the pore length and  $D_w$  is the diffusion coefficient of a single water molecule in the pore (Finkelstein, 1987). Using  $L = 4 \text{ nm}$  and  $D_w = 2.4 \times 10^{-5} \text{ cm}^2/\text{s}$ ,  $r_o$  is  $0.19 \text{ nm}$ . This small diameter is consistent with the exclusion of urea and NaCl from the proximal tubule water channel (Pearce and Verkman, 1989; Shi et al., 1991) and with the low urea, proton and ion permeabilities reported here.

The function of plasma membrane water transporters in kidney proximal tubule and thin descending limb is to transport large volumes of fluid from lumen-to-blood across the tubule epithelium in response to small osmotic gradients. It is not known whether there is transcriptional regulation of CHIP28k message in these tubule segments. Because osmotic disequilibrium does not exist in proximal tubule and thin descending limb, it is unlikely that precise regulation of plasma membrane water permeability in these tubule segments occurs. However, the induction of osmotic disequilibrium by pharmacological inhibition of the water transporter may be of clinical significance. The decreased proximal reabsorption of salt and volume would overload the distal nephron, leading to a salt and water diuresis. Inhibition of CHIP28k water channel function, in conjunction with inhibition of salt transport in distal nephron by conventional diuretics such as furosemide, might be of use in the treatment of a variety of clinical disorders including hypertension, congestive heart failure and the refractory edema states of cirrhosis and the nephrotic syndrome.

We thank Mr. Oliver Baker for performing the two-electrode voltage clamp measurements.

This work was supported by grants DK35124 and HL42368 from the National Institutes of Health, a grant-in-aid from the American Heart Association and a grant from the National Cystic Fibrosis Foundation. Dr. Zhang is a fellow of the American Heart Association, California chapter. Dr. Hasegawa is a fellow of the National Kidney Foundation. Dr. Skach is a recipient of Physician-Scientist award CA01614 from the National Institutes of Health. Dr. Verkman is an established investigator of the American Heart Association.

Received for publication 8 July 1992 and in revised form 9 October 1992.

## References

Biber, J., B. Stieger, W. Haase, and H. Murer. 1981. A high yield preparation for rat kidney brush border membranes. *Biochim. Biophys. Acta.* 647:

- 169–176.
- Brown, D. 1989. Membrane recycling and epithelial cell function. *Am. J. Physiol.* 256:F1–F12.
- Denker, B. M., B. L. Smith, F. P. Kuhajda, and P. Agre. 1988. Identification, purification, and partial characterization of a novel  $M_r$  28,000 integral membrane protein from erythrocytes and renal tubules. *J. Biol. Chem.* 263:15634–15642.
- Finkelstein, A. 1987. Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes: Theory and Reality. John Wiley & Sons, Inc., New York. 42–58.
- Fischbarg, J., K. Kunyan, J. C. Vera, S. Arant, S. Silverstein, J. Loike, and O. M. Rosen. 1990. Glucose transporters serve as water channels. *Proc. Natl. Acad. Sci. USA.* 87:3244–3247.
- Handler, J. S. 1988. Antidiuretic hormone moves membranes. *Am. J. Physiol.* 255:F375–F382.
- Harris, H. W., K. Strange, and M. Zeidel. 1991. Current understanding of the cellular biology and molecular structure of the antidiuretic hormone-stimulated water transport pathway. *J. Clin. Invest.* 91:1–8.
- Harris, H. W., J. B. Wade, and J. S. Handler. 1988. Identification of specific apical membrane polypeptides associated with the antidiuretic hormone-elicited water permeability increase in the toad urinary bladder. *Proc. Natl. Acad. Sci. USA.* 85:1942–1946.
- Hasegawa, H., W. Skach, O. Baker, M. C. Calayag, V. Lingappa, and A. S. Verkman. 1992. A multi-functional aqueous channel formed by CFTR. *Science (Wash. DC).* 258:1477–1479.
- Hasegawa, H., R. Zhang, A. Dohrman, and A. S. Verkman. 1993. Tissue localization of the CHIP28 water channel by *in situ* hybridization. *Am. J. Physiol.* In press.
- Imai, M., Y. Yasoshimi, and K. Yoshitomi. 1990. Mechanism of water transport across the upper portion of the descending limb of long-looped nephrons of hamster. *Pflügers Arch. (Eur. J. Physiol.)* 415:620–631.
- Kuwahara, M., L.-B. Shi, F. Marumo, and A. S. Verkman. 1991. Transcellular water flow modulates water channel exocytosis and endocytosis in kidney collecting tubule. *J. Clin. Invest.* 88:423–429.
- Macey, R. I. 1984. Transport of water and urea in red blood cells. *Am. J. Physiol.* 246:C195–C203.
- Melton, D. A., P. A. Krieg, M. R. Bebagliati, T. Maniatic, K. Zinn, and M. R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacterial SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.
- Meyer, M. M., and A. S. Verkman. 1987. Evidence for water channels in proximal tubule cell membranes. *J. Memb. Biol.* 96:107–119.
- Pardue, M. L. 1988. *In situ* hybridization. In *Nucleic Acid Hybridization: A Practical Approach*. B. D. Hames and S. J. Higgins, editors. Oxford Press. Washington, DC. 179–202.
- Pearce, D., and A. S. Verkman. 1989. NaCl reflection coefficients in proximal tubule apical and basolateral membrane vesicles: measurement by induced osmosis and solvent drag. *Biophys. J.* 55:1251–1259.
- Perara, E., R. E. Rothman, and V. R. Lingappa. 1986. Uncoupling translocation from translation: implications for transport of proteins across membranes. *Science (Wash. DC).* 232:348–352.
- Preston, G. M., and P. Agre. 1991. Isolation of the cDNA for erythrocyte integral membrane protein of 28-kilodaltons - member of an ancient channel family. *Proc. Natl. Acad. Sci. USA.* 88:11110–11114.
- Preston, G. M., T. P. Carroll, W. B. Guggino and P. Agre. 1992. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science (Wash. DC).* 256:385–387.
- Sabolic, I., G. Valenti, J. M. Verbavatz, A. N. van Hoek, A. S. Verkman, D. A. Ausiello, and D. Brown. 1992. Localization of the CHIP28 water channel in rat kidney. *Am. J. Physiol.* 263:C1225–C1233.
- Sabolic, I., L.-B. Shi, D. Brown, D. A. Ausiello, and A. S. Verkman. 1992. Proteinases inhibit H-ATPase and Na-H exchange but not water transport in apical and endosomal vesicles from rat proximal tubule. *Biochim. Biophys. Acta.* 1103:137–147.
- Shi, L.-B., Y.-X. Wang, and A. S. Verkman. 1990. Regulation of the formation and water permeability of endosomes from toad bladder granular cells. *J. Gen. Physiol.* 96:789–808.
- Shi, L.-B., K. Fushimi, and A. S. Verkman. 1991. Solvent drag measurement of transcellular and basolateral membrane NaCl reflection coefficient in mammalian proximal tubule. *J. Gen. Physiol.* 98:379–398.
- Smith, B. L., and P. Agre. 1991. Erythrocyte  $M_r$  28,000 transmembrane protein exists as a multisubunit oligomer similar to channel proteins. *J. Biol. Chem.* 266:6407–6415.
- Tsai, S.-T., R. Zhang, and A. S. Verkman. 1991. High channel-mediated water permeability in rabbit erythrocytes: characterization in native cells and expression in *Xenopus* oocytes. *Biochemistry.* 30:2087–2092.
- Van Heeswijk, M. P. E., and C. H. van Os. 1986. Osmotic water permeability of brush border and basolateral membrane vesicles from rat renal cortex and small intestine. *J. Membr. Biol.* 92:183–193.
- Van Hoek, A. N., M. Wiener, S. Bicknese, L. Miercke, R. Stroud and A. S. Verkman. 1993. Secondary structure analysis of purified CHIP28 water channel by CD and FTIR spectroscopy. *Biophys. J.* In press.
- Van Hoek, A. N., M. D. de Jong, and C. H. van Os. 1990. Effect of dimethylsulfoxide and mercurial sulfhydryl reagents on water and solute permeability of rat kidney brush border membranes. *Biochim. Biophys. Acta.* 1030:203–210.

- Van Hoek, A. N., M. L. Hom, L. H. Luthjens, D. M. de Jong, J. A. Dempster, and C. H. van Os. 1991. Functional unit of 30 kDa for proximal tubule water channels as revealed by radiation inactivation. *J. Biol. Chem.* 266:16633-16635.
- Van Hoek, A. N., L. H. Luthjens, M. L. Hom, C. H. van Os, and J. A. Dempster. 1992. A 30 kDa functional size for the erythrocyte water channel determined *in situ* by radiation inactivation. *Biochem. Biophys. Res. Commun.* 184:1331-1335.
- Van Hoek, A. N., and A. S. Verkman. 1992. Functional reconstitution of the isolated erythrocyte water channel CHIP28. *J. Biol. Chem.* 267:18267-18269.
- Verbavatz, J. M., D. Brown, A. N. van Hoek, and A. S. Verkman. 1992. Freeze fracture and rotary shadowing of the CHIP28 water channel in liposomes. *J. Am. Soc. Neph.* 3:800.
- Verbavatz, J. M., G. Calamita, J. S. Hugon, and J. Bourguet. 1989. Isolation of large sheets of apical material from frog urinary bladder epithelial cells by freeze-fracture. *Biol. Cell.* 66:91-97.
- Verkman, A. S. 1992. Water channels in cell membranes. *Annu. Rev. Physiol.* 54:97-108.
- Verkman, A. S. 1987. Passive H<sup>+</sup>/OH<sup>-</sup> permeability in epithelial brush border membranes. *J. Bioenerg. Biomembr.* 19:481-493.
- Verkman, A. S., W. Lencer, D. Brown, and D. A. Ausiello. 1988. Endosomes from kidney collecting tubule contain the vasopressin-sensitive water channel. *Nature (Lond.)*. 333:268-269.
- Verkman, A. S., K. Skorecki, and D. A. Ausiello. 1984. Radiation inactivation of oligomeric enzyme systems: theoretical considerations. *Proc. Natl. Acad. Sci. USA.* 81:150-154.
- Verkman, A. S., P. Weyer, D. Brown, and D. A. Ausiello. 1989. Functional water channels are present in clathrin coated vesicles from bovine kidney but not from brain. *J. Biol. Chem.* 264:20608-20613.
- Verkman, A. S., and K. Wong. 1987. Proton NMR measurement of diffusional water permeability in suspended renal proximal tubule. *Biophys. J.* 51:717-723.
- Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein purification. *In Methods of Enzymology.* Academic Press. New York. 84-93.
- Ye, R., L.-B. Shi, W. L. Lencer, and A. S. Verkman. 1989. Functional colocalization of water channels and proton pumps on endocytic vesicles from proximal tubule. *J. Gen. Physiol.* 93:885-902.
- Zhang, R., S. Alper, B. Thorens, and A. S. Verkman. 1991. Evidence from oocyte expression that the erythrocyte water channel is distinct from band 3 and the glucose transporter. *J. Clin. Invest.* 88:1553-1558.
- Zhang, R., K. Logee, and A. S. Verkman. 1990. Expression of mRNA coding for kidney and red cell water channels in *Xenopus* oocytes. *J. Biol. Chem.* 265:15375-15378.
- Zhang, R., and A. S. Verkman. 1991. Water and urea transport in *Xenopus* oocytes: expression of mRNA from toad urinary bladder. *Am. J. Physiol.* 260:26-34.