Localization and Functional Analysis of CHIP28k Water Channels in Stably Transfected Chinese Hamster Ovary Cells*

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CHIP28 is a major water transporting protein in erythrocytes and plasma membranes in kidney proximal tubule and thin descending limb of Henle. Chinese hamster ovary cells were stably transfected with the coding sequence of cloned rat kidney CHIP28k using expression vectors containing cytomegalovirus or Rous sarcoma virus promoters. Clonal cell populations expressed a 1.3-kilobase mRNA on Northern blot probed by CHIP28k cDNA and a 28-kDa protein on immunoblot probed by a polyclonal CHIP28 antibody. The clone with greatest expression produced ~8 x 10⁶ copies of CHIP28k protein/cell. Plasma membrane osmotic water permeability (P₀), measured by stopped-flow light scattering, was 0.004 cm/s in control (vector-transfected) cells (10 °C) and 0.014 cm/s in the CHIP28k-transfected cells; this difference correlated with CHIP28k being expressed in transfected cells. P₀ in CHIP28k-transfected cells had an activation energy of 4.9 kcal/mol and was reversibly inhibited by HgCl₂. CHIP28k expression did not affect the transport of protons and the small polar non-electrolytes urea and formamide. CHIP28k immunoreactivity and function was then determined in subcellular fractions. P₀ in 6-carboxyfluorescein-labeled endocytic vesicles, measured by a stopped-flow fluorescence quenching assay, was 0.002 cm/s (control cells) and 0.011 cm/s (CHIP28k-transfected cells); P₀ in transfected cells was inhibited by HgCl₂. Immunoblotting of fractionated endoplasmic reticulum, Golgi, and plasma membranes revealed high densities of CHIP28k (~5000 monomers/μm² in plasma membrane) with different glycosylation patterns; functional water transport activity was present only in Golgi and plasma membrane vesicles. Antibody detection of CHIP28k by confocal fluorescence microscopy and immunogold electron microscopy revealed localization to plasma membrane and intracellular vesicles. These studies establish a stably transfected somatic cell line that strongly expresses functional CHIP28k water channels. As in the original proximal tubule cells, the expressed CHIP28k protein is a selective water channel that is functional in endocytic vesicles and the cell plasma membrane.

An abundant hydrophobic 28-kDa protein from human erythrocytes (CHIP28) was recently isolated and cloned (1, 2). The amino acid sequence indicates homology to a class of ancient channel-like proteins which include the major intrinsic protein of lens, related proteins from bacteria, yeast, and plants (3), and a recently cloned protein from kidney collecting duct (4). CHIP28 functions as a selective water channel in the erythrocyte and selected tubule segments of the mammalian nephron. Expression of mRNA encoding CHIP28 in Xenopus oocytes increased water but not ion permeability (5, 6), and proteoliposomes reconstituted with purified CHIP28 protein had high water but not proton and urea permeabilities (6-8). Antibody staining localized CHIP28 to plasma and intracellular membranes of constitutively water-permeable tissues in rat kidney, including proximal tubule and thin descending limb of Henle (9, 10). In situ hybridization confirmed this localization and indicated a highly selective tissue distribution of mRNA encoding CHIP28 in selected epithelial and/or endothelial cells in lung, intestine, brain, eye, and other tissues (6, 11).

In epithelial cells in kidney proximal tubule, high plasma membrane water permeability is important for the near isosmotic reabsorption of water filtered by the glomerulus. Functional studies indicate the presence of a water transporting protein on apical and basolateral plasma membranes (12, 13), as well as on apically derived endocytic vesicles (14) and clathrin-coated vesicles (15). Intact tubule and cell-free vesicle measurements showed that water permeability in these membranes is high, weakly temperature dependent, and inhibited by mercurial sulphydryl-reactive compounds (for review, see Refs. 16, 17). An antisense CHIP28 oligonucleotide blocked the increase in water permeability in oocytes expressing heterologous mRNA from kidney cortex (6), suggesting that CHIP28 (or homologous proteins) is a major water transporter in kidney proximal tubule.

The purpose of this study was to establish a stably transfected somatic cell line expressing functional CHIP28 water channels and to investigate the stage of biogenesis in which CHIP28 protein attains functional maturity. In plasma membranes, it is believed that individually functional CHIP28 monomers (18-20) are assembled in tetramers (1, 21) in which ~50% of the monomeric subunits are glycosylated. Because of the small single channel water permeability of CHIP28 (~10⁻¹⁰ cm²/s, Ref. 5) and the relatively high endogenous water permeability of biological membranes (~10⁻⁹ cm²/s), a high membrane density of functional water channels (>100/μm²) was required in the somatic cell to increase water permeability significantly. In our studies, high expression of CHIP28 was accomplished in CHO-K1 (wild type) cells. The transporting characteristics of the expressed CHIP28 protein were similar to those in the original kidney cells. Interestingly, as observed in the native kidney proximal tubule, CHIP28 water channels were functional in both endocytic vesicles and...
the cell plasma membrane. CHIP28 water channels were also localized in endoplasmic reticulum and Golgi, but functional only in Golgi. The results establish the first cultured somatic cell line for study of water channel biology.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The cDNA encoding rat kidney water channel CHIP28k in plasmid pSP64.CHIP28k (6) was used as template to amplify the 807 base pair coding region and introduce HindIII (5') and XbaI (3') restriction sites. The polypeptide chain reaction fragment was filled-in with Klenow DNA polymerase I. The HindIII and XbaI cut fragment was ligated into mammalian expression vectors pRC/CMV1 and pRc/RSV (Invitrogen) at the HindIII and XbaI sites respectively. The CHIP28k sequences in the plasmid constructs were confirmed by DNA double-stranded sequencing following denaturation by the dye-oxide chain-termination method using a Sequenase sequencing kit (U. S. Biochemical Corp.).

**Cell Culture and Transfection**—CHO-K1 (wild type) cells (obtained from University of California, San Francisco Cell Culture Facility) were grown in Ham's Nutrient Mix supplemented with 10% fetal calf serum at 37 °C in 5% CO₂. Transfection was carried out by use of Lipofectin (22). Cells were plated at a density of 5 × 10⁶ cells/60-mm diameter dish 12 h before transfection. The cells were washed three times just before transfection with Opti-MEM1 media (Life Technologies, Inc.) diluted into 1 ml of serum-free Opti-MEM1 media, then combined with 1 ml of serum-free Opti-MEM1 media containing 10 μg of each recombinant plasmid (or vector only) and incubated at room temperature for 15 min. The mixture was then added to the washed CHO-K1 cells. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 12 h, washed twice with Ham's Nutrient Mix, and incubated for 24 h in 4 ml of Ham's Nutrient Mix supplemented with 10% fetal calf serum. Cells grown on each 60-mm dish were trypsinized and transferred to three 100-mm diameter dishes. Selection of cells containing stably integrated cDNA was accomplished by adding G418 (G418, Life Technologies, Inc.) to the growth media at a concentration of 500 μg/ml. After selection for 10–14 days, G418-resistant cell clones were isolated and transferred to separate culture dishes for expansion and analysis.

**Northern Blot Analysis**—Total RNA was isolated from ~10⁶ cells of each G418-resistant clone (and mock-transfected CHO-K1 cells) by using SDS-EDTA lysis followed by phenol/chloroform extraction. 50 μg of each total RNA was resolved on a formaldehyde-agarose gel and blotted overnight onto a nylon membrane. The RNA was cross-linked to the membrane by UV light, prehybridized for 2 h in 50% formamide, 2% SDS, and 100 μg of denatured salmon sperm DNA at 50 °C, and hybridized overnight with 10⁶ counts/min/ml of CHIP28 cDNA probe (corresponding to the 807-base pair coding sequence) labeled with [α-32P]dCTP (Amersham Corp.) by random priming (Bethesda Research Laboratories). After hybridization, the membrane was washed twice with 5 × SSC, 0.1% SDS at room temperature, twice at 50 °C for 15 min each, and once with 0.1 × SSC, 0.1% SDS at 50 °C for 30 min. Hybridization was visualized by autoradiography. The same membrane was washed twice with boiling water and hybridized with a rat α-tubulin cDNA probe by the same procedure.

**Immunoblot of CHIP28 Protein**—Cells grown on a 100-mm diameter dish were treated with 50 μM EDTA for 2 min, washed once with PBS, and centrifuged. The supernatant was removed and 1 ml of 1 × sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, 0.01% bromphenol blue, pH 6.8) was added to the cell pellet. Samples were heated to 80 °C for 5 min, sonicated for 1 min, and then centrifuged at 10,000 × g for 10 min. The supernatants (40 μl) were resolved on 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin in TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 20 min at room temperature and incubated overnight with a rabbit polyclonal antibody (1:1000 dilution in blocking solution, Ref. 9). The membrane was washed four times with TBS-T, incubated with an anti-rabbit IgG-alkaline phosphatase conjugate antibody (Promega, 1:5000 dilution) and developed with 5-bromo-4-chloro-3-indolylphosphate (16 μg/ml) and nitroblue tetrazolium (0.33 μg/ml) or detected by enhanced chemiluminescence (Amersham Corp.).

**Immunocytochemistry**—For antibody staining, CHO-K1 cells were cultured on glass coverslips overnight, fixed with 4% formaldehyde in PBS for 20 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. The immune serum containing polyclonal CHIP28k antibody (and preimmune serum control) was incubated in PBS containing 3% bovine serum albumin and 0.2% Triton X-100 for 1 h at room temperature and then washed four times with PBS containing 1% Triton X-100. Cells were then incubated with the secondary FITC-conjugated goat anti-rabbit IgG (1:20 dilution) for 1 h and washed four times in PBS containing 1% Triton X-100. Slides were viewed in a Leitz-Technica Instruments epifluorescence confocal microscope with a cooled CCD camera detector. Confocal images were obtained with a ×60 objective (Nikon, oil immersion, numerical aperture 1.4) in which the measured z-axis resolution of the confocal optics was 1.2 μm. In separate experiments, fluorescently stained endocytic vesicles were labeled and examined as described previously (23).

For immunogold electron microscopy, cells cultured on plastic were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde and washed three times with 0.1% sodium cacodylate and 1.5% sodium carbonate in PBS, preincubated with PBS containing 1% albumin for 20 min, and then incubated with the 1:4000 dilution of immune serum for 90 min. Grids were washed in PBS and incubated with a 1:50 dilution of 15-nm protein A-gold in albumin-PBS. Grids were washed and fixed for 20 min in 1% glutaraldehyde in PBS. The grids were then washed with water, stained, and embedded in 0.2% uranyl acetate and 0.5% methylcellulose in water for 10 min, and dried. Sections were observed and photographed on a Philips CM10 electron microscope.

**Assays for Plasma Membrane Water, Solute, and Proton Transport**—Water permeability (Pw, cm/s) was measured in freely suspended CHO-K1 cells by a light scattering method (12, 15) using a Hi-Tech SF51 stopped-flow apparatus. The instrument dead time was 1.6 ms and sample temperature was controlled by a circulating water bath. Cells from several 100-mm diameter plastic dishes were suspended in PBS by incubation in Ca²⁺/Mg²⁺-free PBS containing 50 mM EDTA for 2 min at 23 °C. Cells were washed three times in PBS and suspended at a concentration of ~2 × 10⁶ cells/ml. 0.1 ml of the cell suspension was mixed in 1 ml with an equal volume of PBS containing 500 mM sucrose to give a 250 mM inwardly directed osmotic gradient. The osmotic gradient caused water efflux, cell shrinkage, and an increased light scattering. The time course of scattered light intensity at 530 nm was recorded. Measurements were performed five to eight times in each sample for signal averaging. Data were fitted to a hyperbolic function; water transport rates (Aₐ, nm/s) were calculated from the initial slope curve normalized to the total signal amplitude. Pₐ values were calculated from Aₐ and the cell surface-to-volume ratio (5450 cm⁻², calculated from average cell diameter of 11 μm measured by phase-contrast microscopy) as described previously (12, 15). Cell permeability (Pₑ, cm/s) for the small polar solutes urea and formamide was determined from the time course of scattered light intensity in response to a 250 mM inwardly directed osmotic gradient. The solute gradient caused faster cell shrinkage due to osmotic water efflux, followed by slower cell swelling due to solute and water influx.

Passive proton permeability was measured from the kinetics of intracellular acidification in response to a sudden decrease in solution pH from 7.4 to 6.0. Freshly suspended CHO-K1 cells were loaded with the fluorescent pH indicator 2,7-bis-(2-carboxyethyl)-5(6)-and 6-carboxyfluorescein (BCECF) by a 20-min incubation with 2 μM BCECF-acetoxyethylmethylster at 37 °C. Extracellular fluorophore was removed by two washes. The BCECF-loaded cells were suspended in a buffer consisting of 50 mM NaCl, 10 mM KCl, 5 mM potassium phosphate, pH 7.4, containing 2 μM valinomycin. The membrane potential was depolarized and the K permeability was increased so that the rate-limiting step for dissipation of an imposed pH gradient was the passive proton permeability (24). The cell suspension was incubated with the stopped buffer and acidified with an equal volume of the same buffer (titrated to pH 2.7) so that pH 6.0 was reached. The time course of BCECF fluorescence (excitation 490 nm, emission
Stable Transfection of CHIP28k Water Channels

Expression of CHIP28k in Transfected Cells—Clonal cell lines derived from stably transfected CHO-K1 cells were expanded and examined for CHIP28k mRNA and protein. Fig. 1A shows a Northern blot of RNA isolated from a series of clonal cell lines probed with 32P-labeled CHIP28k and α-tubulin cDNAs. There was no expression of CHIP28k mRNA in cells transfected with vector alone (mock-transfected) and variable levels of expression in cells transfected with the coding region of CHIP28k. Expression was variable with the CMV vector, but consistently high with the RSV vector. Expression of α-tubulin (control) was observed in every cell line.

RESULTS

Expression of CHIP28k in CHO-K1 cells. A, Northern blot. 150 μg of total RNA from mock-transfected cells (CMV vector alone) and 50 μg of total RNA from each CHIP28-transfected cell was resolved on a agarose gel and blotted with CHIP28 and α-tubulin cDNA probes (see “Experimental Procedures”). RNA from six different clones transfected with CMV vector and four different clones with RSV vector are shown. B, Western blot. Total protein from 3 cm² of confluent CHIP28-transfected (clone CMV-4, center lane) and mock-transfected (right lane) CHO-K1 cells was electrophoresed on 12% SDS-polyacrylamide gel electrophoresis and blotted with a rabbit anti-CHIP28 polyclonal antibody (see “Experimental Procedures”). The left lane contains 0.5 μg of CHIP28 protein purified from human erythrocytes (7).

Fig. 1B shows a Western blot of total protein from the CHO-K1 cell line CMV-4, probed with a rabbit polyclonal antibody raised against purified human CHIP28. The CHIP28k-transfected cells (center lane) showed a sharp band at 28 kDa, corresponding to non-glycosylated CHIP28k (32), and a broad band at 35–50 kDa, corresponding to glycosylated CHIP28k. These bands were absent in the mock-transfected cells (right lane). The position of the 28 kDa band and the glycosylation were similar to that observed for purified erythrocyte CHIP28 (left lane). Quantitative immunoblotting of the CHIP28k-transfected cell homogenate using a series of CHIP28 standards indicated that the CMV-4 cell line produced ~0.4 pg of CHIP28k protein (8 × 10⁵ copies) per cell. These studies indicate the expression of CHIP28k mRNA and immunoreactive protein only in the stably transfected CHO-K1 cells.

The morphology of the CMV-4 stably transfected cells (more than five passages after cloning) was not different from that of the mock-transfected cells as observed by phase-contrast light microscopy and transmission electron microscopy (not shown). freshly suspended mock- and stably transfected cells were spherical with an average diameter of 11 μm. The growth characteristics of the mock- and stably transfected cells were not different as assayed by [3H]thymidine incorporation (1 μCi/well of 24-well plate; relative 8 h incorporation: mock, 1.0; CHIP28k, 1.2) and cell density (in cells/cm²) at 24 h (mock 5.7 × 10⁵; CHIP28k, 6.2 × 10⁵) and 48 h
patterns of endocytic uptake of 6CF and FITC-transferrin in density of 2.5 cells changed after five to eight passages, whereas the transduced cells having lower levels of CHIP28k expression could be passed >15 times without notable change.

**Cell Osmotic Water Permeability—**A stopped-flow light scattering method was used to examine CHIP28k function in the plasma membrane of transfected cells. The adherent cells were freshly suspended by agitation in an EDTA-containing solution. Fig. 2, A and B, show the time course of scattered light intensity in response to a sudden increase in extracellular osmolality from 300 to 550 mOsm. The osmotic gradient caused water efflux, cell shrinkage, and an increase in scattered light intensity. The data are shown using three times scales to follow the full kinetics of cell shrinkage. $P_i$ in the mock-transfected cells was $0.0039 \pm 0.0002$ cm/s (S.E., $n = 5$ separate sets of measurements, each consisting of the average of five to eight individual curves) at 10 °C. $P_i$ in the mock-transfected cells was not affected by HgCl$_2$, $\beta$-mercaptoethanol, or dimethyl sulfoxide (Fig. 2A and Table I). The cells transfected with CHIP28k (Fig. 2B) had a remarkably increased $P_i$ as shown by the increase in initial slope. At 10 °C, the cells used in Fig. 2B, which had the highest CHIP28k mRNA expression by Northern analysis (clone CMV-4), had a $P_i$ of $0.014 \pm 0.001$ cm/s ($n = 5$). Although a clonal cell population was used for this experiment, biexponential analysis indicated that 68% of the signal arose from cells having high $P_i$; the remaining cells with lower apparent $P_i$ may have been damaged in the suspension procedure. (For this reason, $P_i$ was measured in isolated surface membrane vesicles, see below.) The level of CHIP28k expression for several clonal cell populations (mock, CMV-1, CMV-3, RSV-1, and CMV-4; relative mRNA expression by densitometry: 0, 0.04, 0.22, 0.46, 1), correlated reasonably well with measured plasma membrane $P_i$ (in cm·s$^{-1}$ at 10 °C: 3.9, 4.5, 6.9, 10, and 14, respectively).

Two additional characteristics of the CHIP28k water channel in native tissues are inhibition by mercurials and a weak temperature dependence (16, 17). Fig. 2B shows that incubation of the CHIP28k-transfected cells with 20 mM HgCl$_2$ for 5 min strongly inhibited the increase in $P_i$ associated with CHIP28k (54 ± 3% inhibition, $n = 3$). The inhibition by HgCl$_2$ was partially reversed (to 21 ± 5% inhibition) by post-treatment with $\beta$-mercaptoethanol. Fig. 2B also shows that dimethyl sulfoxide inhibited the CHIP28k-associated increase in $P_i$, but had no effect on $P_i$ in mock-transfected cells. The cAMP agonists forskolin (25 μM) and chlorophenylthio-cAMP (0.5 mM) did not affect $P_i$ in mock- or CHIP28k-transfected cells (not shown). Fig. 2C shows an Arrhenius plot for the temperature dependence of $P_i$. The activation energy ($E_a$), given by the slope of the Arrhenius plot (Fig. 2C), was 4.9 ± 0.6 kcal/mol for cells expressing CHIP28k and 2.0 ± 0.8 kcal/mol for the mock-transfected cells. Taken together, the high $E_a$ for the inhibition by HgCl$_2$ and dimethyl sulfoxide, and the low $E_a$ for the expressed CHIP28k protein in CHO-K1 cells is functionally similar to that in the original tissue.

To investigate whether the expressed CHIP28k protein transported small solutes, measurements of urea, formamide, and proton permeability were carried out in the mock- and CHIP28k-transfected cells. Table I summarizes the results. Although water permeability differed in the mock- and stably transfected cell lines, there was no systematic difference in the permeabilities of the small solutes urea and formamide. The passive proton permeability, measured from the rate of disappearance of a preformed pH gradient, was not different in the mock- and CHIP28k-transfected cells. These results indicate that CHIP28k is a selective water transporter that excludes small solutes.
TABLE I

Permeability properties of transfected CHO-K1 cells

| Permeability (mean ± S.E., n = 3–5 separate sets of measurements, each consisting of the average of five to eight individual curves) were measured as described under “Experimental Procedures.” The CHIP28k-transfected cell line was clone CMV-4 and the mock-transfected cell line was established by transfection with the empty CMV vector. Water transport experiments were carried out at 10 °C as described in the legend to Fig. 2.

|                 | Units       | Mock-transfected | CHIP28k-transfected |
|-----------------|-------------|------------------|---------------------|
| Water           | cm/s        | 0.0039 ± 0.0002  | 0.014 ± 0.001       |
| +HgCl₂          | cm/s        | 0.0037 ± 0.0003  | 0.0085 ± 0.001      |
| +HgCl₂ + ME     | cm/s        | 0.0038 ± 0.0003  | 0.011 ± 0.001       |
| Dimethyl sulfoxide | cm/s        | 0.0040 ± 0.0002  | 0.0045 ± 0.0006     |
| Proton pH       | units/s     | 0.85 ± 0.1       | 0.95 ± 0.1          |
| Urea            | 10⁻⁴ cm/s   | <1               | <1                  |
| Formamide       | 10⁻⁴ cm/s   | 24 ± 5           | 29 ± 8              |

Endosome Water Permeability—Osmotic water permeability was measured in endosomes derived from the CHO-K1 cells. Endosomes were labeled with the membrane-impermeant fluorophore 6CF by fluid-phase endocytosis. Water permeability was assayed in a crude microsomal fraction containing the fluorescently labeled endosomes using a stopped-flow fluorescence quenching technique. The osmolyte of buffer bathing the microsomes was suddenly increased from 60 to 160 mOsm, causing osmotic water efflux, microsome shrinkage, and 6CF fluorescence self-quenching. The fluorescence signal originated selectively from the fluorescently labeled endosomes, and not from the majority of nonfluorescent microsomes. Fig. 3 shows that the rate of fluorescence decrease was remarkably more rapid in endosomes derived from CHIP28k-transfected CHO-K1 cells (Fig. 3B, upper curve) than from mock-transfected cells (Fig. 3A, upper curve). *P* values were 0.0021 cm/s (CHIP28k-transfected) and 0.011 ± 0.001 cm/s (CHIP28k-transfected cells). Analysis of pre-exponential factors of the biexponential fit indicated that >60% of the fluorescently labeled vesicles contained functional water channels. In control studies in which endocytosis was inhibited (33), the CHIP28k-transfected cells were labeled with 6CF at 4 °C (instead of 37 °C), or at 37 °C in the presence of NaN₃. Fig. 3B (bottom curves) shows that the signal amplitude was nearly zero, suggesting that generation of the fluorescence signal required endocytosis.

The inhibition properties and temperature dependence of endosome *P*₇ were measured. The increase in *P*₇ in endosomes derived from CHIP28k-transfected cells was inhibited by 92 ± 6% (*n* = 2) by 0.6 mM HgCl₂, whereas *P*₇ in endosomes derived from the mock-transfected cells was unaffected (inhibition 8 ± 9%, Fig. 3). Similar inhibition of *P*₇ in CHIP28k-transfected cells, but not mock-transfected cells, was observed with dimethyl sulfoxide. When measured at 21 °C, endosome *P*₇ values were 0.0014 cm/s (CHIP28k-transfected) and 0.0039 cm/s (mock-transfected). Assuming a linear Arrhenius relation, calculated *E*ₐ values were 4.2 kcal/mol (CHIP28k-transfected) and 9.6 kcal/mol (mock-transfected). These results indicate the presence of functional water channels in endosomes derived from the CHIP28k-transfected, but not from the mock-transfected CHO-K1 cells.

CHIP28k Localization and Function in Isolated Membrane Fractions—To examine the functional maturation of CHIP28k, cell homogenates were fractionated by sucrose gradient centrifugation for immunoblot and functional analysis. Fig. 4A shows the specific activities of marker enzymes for plasma membranes, Golgi and endoplasmic reticulum. The fractionation pattern was not different for the mock- and CHIP28k-transfected cells and is similar to previous reports in CHO cells (28). Note that α-mannosidase is not a highly specific Golgi marker; Golgi vesicles are primarily present in fractions 9–11 and should be absent in fractions 1–8. The alkaline phosphodiesterase activity in fractions 1 and 2 is due to the presence of lysosomes. Fig. 4B is an immunoblot probed with the anti-CHIP28 antibody. The endoplasmic reticulum vesicles showed mainly the 28 kDa band (arrow a) with some intermediate form (arrow b), whereas the Golgi and plasma membranes show a glycosylation pattern (arrow c) similar to that observed in native erythrocyte and kidney membranes. Fig. 4C shows a series of stopped-flow light scattering measurements of osmotic water permeability on the enriched vesicle fractions. The plasma membranes from CHIP28k-
transfected cells had a very high $P_f$ (0.026 cm/s) that was inhibited by >95% by HgCl$_2$ to levels observed for plasma membranes isolated from the mock-transfected cells. The analysis indicated that >95% of plasma membrane vesicles were highly water permeable. Water transport in plasma membranes from mock-transfected cells was not inhibited by HgCl$_2$ (not shown). Quantitative immuno blotting showed the presence of 78 µg of CHIP28k (glycosylated + non-glycosylated) per mg total plasma membrane protein. Water transport was also high ($P_f$ 0.015 cm/s) and HgCl$_2$ inhibitable in the Golgi fraction from CHIP28k-transfected cells; $P_f$ was low (0.0013 cm/s) and HgCl$_2$ insensitive in the Golgi fraction from the mock-transfected cells. In contrast, immunoreactive CHIP28k protein was present in the endoplasmic reticulum fraction, but not functional as shown by the low $P_f$ that was similar to $P_f$ in the endoplasmic reticulum fraction from mock-transfected cells, and the lack of inhibition by HgCl$_2$. These studies indicate that CHIP28k attains functional competence in the Golgi.

**Immunolocalization of CHIP28k**—Fluorescent antibody staining experiments were carried out to determine the cellular location of expressed CHIP28k. In non-permeabilized fixed cells, there was little cell staining, consistent with immunoelectron microscopy studies suggesting that the antibody binds predominantly to a cytoplasmic epitope of CHIP28k (9). Permeabilized CHIP28k-transfected cells were strongly stained (Fig. 5B) compared to the mock-transfected cells (Fig. 5A). The staining of the mock-transfected cells was similar to that observed for the CHIP28k-transfected cells incubated with preimmune serum (not shown).

To determine the subcellular location of CHIP28k, cells were viewed with high magnification confocal optics. Fig. 5, C and D, are confocal micrographs of permeabilized adherent cells where the focal plane was near the base of the cells (Fig. 5C) and half-way between the cell base and apex (Fig. 5D). Note that the same cell field is shown in Fig. 5, C and D. In Fig. 5C, the plasma membrane was well stained as shown by the linear fluorescence at the cell base. In Fig. 5D, staining of intracellular vesicles was observed in the cytosolic, but not the nuclear compartment. Staining of endocytic vesicles was confirmed by a double label experiment in which many FITC-stained vesicles colocalized with vesicles that had previously internalized tetramethylrhodamine-dextran by fluid-phase endocytosis (not shown). No staining of intracellular vesicles was observed in the CHIP28k-transfected cells using preimmune serum or in the mock-transfected cells.

CHIP28k cellular localization was studied at higher resolution by immunogold labeling. The polyclonal anti CHIP28 antibody did not label mock-transfected cells (Fig. 6A). In contrast, the plasma membrane of CHIP28k-transfected cells was labeled heavily (Fig. 6B). Although most of the gold labeling was located on the plasma membrane, anti-CHIP28 antibodies consistently labeled Golgi (Fig. 6C) and a population of cytoplasmic vesicles (Fig. 6D).

**DISCUSSION**

The purpose of this study was to establish a somatic cell line that stably expressed functional CHIP28k water channels. High levels of CHIP28k mRNA and protein were expressed in CHO-K1 cells that were stably transfected with CHIP28k cDNA in plasmids having CMV or RSV viral promoters. The functional characteristics of the expressed water channel were similar to those in the original tissue. The functional studies supported the conclusion that CHIP28k is a selective water transporting protein. The stably transfected cells expressing CHIP28k should be useful for studies of the functioning, pharmacology, and trafficking of mutant and homologous CHIP28 proteins. In addition, the abundant quantities of expressed functional protein make the transfected CHO-K1 cell system suitable for generation of mutant and homologous proteins for which there is no suitable native tissue source.

There were a number of special concerns for the stable expression of water channels in somatic cells. First, whereas...
permeability. In erythrocytes, kidney cells, and the "high protein is CHIP28. Second, it was not known were incubated with a rabbit anti-CHIP28 polyclonal antibody and expression” CHO-K1 cells studied here, 2-8% of membrane microscopy method has been applied to measure water channel (17) require the expression of large quantities of CHIP28k protein had osmotic gradients and hydrostatic forces, some cell processes, volume changes. It has been proposed that the kinetics of the increase in water permeability in cell plasma membranes factors that limit the volume of water moving through a single water channel (17) require the expression of large quantities of water channels to give a measurable increase in water permeability. In erythrocytes, kidney cells, and the “high expression” CHO-K1 cells studied here, 2-8% of membrane protein is CHIP28. Second, it was not known a priori whether the increase in water permeability in cell plasma membranes and intracellular vesicles would affect cell viability. Although water movement across membranes is always secondary to osmotic gradients and hydrostatic forces, some cell processes, such as fusion and sorting of vesicles, are associated with volume changes. It has been proposed that the kinetics of volume change may be important for vesicle fusion and other intracellular events (34). We found that the transfected CHO cells expressing large quantities of CHIP28k protein had similar morphology, growth characteristics, and vesicular trafficking to the mock-transfected (vector alone) cells.

Another concern in these studies was the functional assay for water channels. There are no good methods to measure rapid volume changes in adherent cells. A light scattering microscopy method has been applied to measure Pi in relatively large round cells (J774 macrophages, Ref. 35), but was unsuccessful for the CHO-K1 cells. Our approach here was to adapt the stopped-flow light scattering method to measure the rate of osmotically induced cell shrinkage of freshly suspended cells. Initial studies in suspended cells obtained by trypsinization of the adherent cultures showed no evidence of functional water channels, probably because of partial digestion of the water channel protein. High water permeability in stably transfected cells was successfully observed when cells were released from the plastic support by EDTA and mechanical agitation. The light scattering method was also used to measure water permeability in isolated subcellular vesicles. The measurement of water transport in small vesicles is not subject to effects of unstirred layers or loss of cell viability during the suspension procedure.

Although a high level of functional CHIP28k protein was expressed in the stably transfected CHO-K1 cells, ~10-fold lower levels were obtained in stably transfected MDCK epithelial cells using the same transfection procedures (data not presented). Further, preliminary attempts to express high levels of CHIP28 by transient transfection of COS cells using vector pCDM8 (Invitrogen) were unsuccessful. The generation of transfected polar epithelial cells lines would be particularly useful for studies of CHIP28k targeting because CHIP28k may be one of the few transporting proteins that is present on both apical and basolateral membranes (9).

The characteristic features of the water transporting pathway in erythrocytes and kidney proximal tubule are a high and selective membrane permeability to water, inhibition of water permeability by mercurials, a weakly temperature-dependent water permeability, and a high ratio of osmotic-to-diffusional water permeability (16, 17). Pi in the stably transfected cells was 0.018 cm/s at 20 °C, similar to that of 0.02 cm/s in human erythrocytes (36) and 0.01-0.04 cm/s in proximal tubule apical and basolateral plasma membranes (12). It is estimated that each CHO-K1 cell contains 1.6 × 10^9 copies of CHIP28k in the plasma membrane based on the density of CHIP28k measured in the surface membrane fraction (4900/μm², see below) and cell surface area (380 μm²). Compared to the total number of immunoreactive CHIP28k molecules/cell of 8 × 10^8 estimated by quantitative immunoblot, it is concluded that significant quantities of CHIP28k are intracellular. This conclusion is supported by the findings of: (a) immunoreactive and functional water channels in Golgi (Fig. 4, (b) functional water channels in the majority of endocytic vesicles (Fig. 3), and (c) immunolocalization of CHIP28k to intracellular vesicles by confocal (Fig. 5) and electron (Fig. 6) microscopy.

The expressed CHIP28k water transporter was functionally similar to that in the original kidney tissue in terms of single channel water permeability, water transport specificity, inhibition pharmacology, and Arrhenius activation energy. The single channel water permeability (Pi) of the CHIP28 water channel was ~5 × 10^-14 cm^3/s at 10 °C in reconstituted proteoliposomes containing purified CHIP28 (7, 8). In the surface membrane fraction isolated from CHIP28k-transfected cells, Pi of the expressed CHIP28k protein was similar (6 × 10^-14 cm^3/s) as calculated from a CHIP28k (monomer) channel density of 4300/μm² (determined by quantitative immunoblot, assuming 50% of membrane weight is protein) and the measured vesicle Pi of 0.026 cm/s at 10 °C. There was no difference in proton, urea, and formamide permeabilities in the mock- and CHIP28k-transfected cells. Water permeability in the CHIP28k-transfected CHO-K1 cells was inhibited by HgCl₂ and dimethyl sulfoxide; the remaining water permeability after inhibition was 0.0045 cm/s, similar to that

**FIG. 5. Immunofluorescence staining of CHIP28k in CHO-K1 cells.** A and B, permeabilized fixed CHO-K1 cells (A, CMV mock-transfected; B, CHIP28k-transfected) grown on glass coverslips were incubated with a rabbit anti-CHIP28 polyclonal antibody and fluorescently stained with an FITC-conjugated goat anti-rabbit IgG (H+L) secondary antibody. Cells were viewed by wide-field epifluorescence microscopy with FITC filter set and imaged by a cooled CCD camera. Illumination intensities and gains were identical in A and B. C and D, confocal epifluorescence micrographs of CHIP28k-transfected cells prepared as in B. Images were obtained at the cell base adjacent to the coverglass (C) and approximately half-way between the cell base and apex (D). The same field is shown in C and D. Magnifications: A and B, × 5000; C and D, × 1000.
of 0.004 cm/s in the mock-transfected CHO-K1 cells. The Arrhenius activation energy for the expressed water channel in CHO-K1 cells was 4.9 kcal/mol, within the range of 2–5 kcal/mol for the erythrocyte and kidney proximal tubule (16, 36). The results suggest that the functional properties of expressed CHIP28 water channels are similar to those in the native erythrocyte and kidney. It was not possible to measure the ratio of osmotic-to-diffusional water permeability in these cells because the diffusional water permeability would be seriously underestimated (and not easily correctable) because of unstirred layer effects.

Immunoblot analysis indicated CHIP28k water channels were present on plasma membrane, endoplasmic reticulum, and Golgi fractions isolated from stably transfected CHO-K1 cells. Whereas little glycosylation was found in the endoplasmic reticulum fraction, the glycosylation pattern of surface membranes was similar to that in CHIP28 from native tissues in which ~50% of monomers are glycosylated. The glycosylation pattern of Golgi vesicles was similar to that in plasma membranes. Functional studies were performed in the plasma membrane, endoplasmic reticulum, and Golgi vesicles by a stopped-flow light scattering technique. Plasma membrane and Golgi fractions had high water permeability that was inhibited by HgCl2; however, CHIP28k was present but not functional in endoplasmic reticulum. These studies indicate that functional maturity of CHIP28k is attained in Golgi. Although site-directed mutagenesis studies indicate that CHIP28k glycosylation is not necessary for its water transporting function (20), there may be other topological or biochemical modifications achieved in Golgi that confer water transporting function.

Functional CHIP28k water channels were demonstrated in endosomes by a fluorescence quenching assay performed on crude microsomes containing fluorescently labeled endosomes. The assay for water transport in endosomes was used previously to demonstrate functional water channels in apically derived endosomes from kidney proximal tubule (14) and from vasopressin-stimulated kidney collecting tubule (26) and toad urinary bladder (27, 33). Water permeability in endosomes isolated from these native cells was high ($P_f$, 0.03–0.1 cm/s), weakly temperature-dependent ($E_a$, 2–5 kcal/mol), and inhibited by HgCl2. Using the same methods, water channels were absent in endosomes derived from a series of established epithelial cell lines derived from renal tubules and amphibian urinary bladder (25). It was found here that water permeability in endosomes derived from mock-transfected CHO-K1 cells was low and not inhibited by HgCl2 and dimethyl sulfoxide, whereas endosomes from CHIP28k-transfected cells had high $P_f$ that was weakly temperature-dependent ($E_a$, 4.2 kcal/mol) and strongly inhibited by HgCl2 and dimethyl sulfoxide. Using a single channel CHIP28k water permeability of $5 \times 10^{-14}$ cm$^3$/s, endosome $P_f$ of 0.011 cm/s, and endosome diameter of 195 nm, it is estimated that each endosome contained 260 functional CHIP28 water channels. The estimated membrane density of water channels in the endosomal membrane (2000/μm$^2$) was less than that of 4300/μm$^2$ calculated for the cell plasma membrane. Analysis of the fluorescence curve shape in Fig. 3B indicated that the majority of fluorescently labeled endosomes in the CHIP28k-transfected cells were water-permeable. These results suggest that CHIP28k is present on endocytic vesicles in the stably transfected cells at a density of ~50% of that found in the cell plasma membrane.

Functional CHIP28k water channels are present on endocytic vesicles in kidney proximal tubule (14). The role(s) of these intracellular water channels is not known, nor is there evidence that water permeability in proximal tubule is regulated by physiological factors. Apical membrane turnover in proximal tubule is very rapid and assumed to be constitutive (37), whereas in collecting duct, water channel trafficking between the cell apical membrane and an intracellular vesicular compartment is regulated by the hormone vasopressin (38). In proximal tubule, the high density of water channels present in intracellular vesicles raises the possibility that
plasma membrane water permeability may, under some conditions, be regulated by physiological factors. Studies of water transport regulation in isolated perfused proximal tubules are required to address this issue.

The stably transfected cell line developed here should be useful in studies of the biochemical and intracellular processing of CHIP28k water channels. Stably transfected cell lines have provided important data about transporting mechanisms and biochemical processing for a number of integral membrane transport proteins, including the cystic fibrosis gene product (CFTR) (39), sodium channel (40), and γ-aminobutyric acid transporter (41). Recently, Lukacs et al. (42) examined the distribution and function of CFTR using CHO-K1 cells that were stably transfected with CFTR cDNA. They obtained functional evidence that CFTR was functional in endocytic vesicles. In C2C12 cells transfected with glucose transporters, the GLUT1 and GLUT4 proteins were also targeted to intracellular vesicles, however insulin did not cause glucose transporter exocytosis (43). The stably transfected cells described here should be useful for identification of the intracellular signals involved in water channel processing and plasma membrane targeting.

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REFERENCES
1. Smith, B. L., and Agre, P. (1991) J. Biol. Chem. 266, 6407-6415
2. Preston, G. M., and Agre, P. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 11110-11114
3. Wintow, G. J., Pisano, M. M., and Chepelevsky, A. B. (1991) Trends Biochem. Sci. 16, 170-171
4. Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Maruno, F., and Sasaki, S. (1983) Nature 301, 549-552
5. Preston, G. M., Carroll, T. P., Guggino, W. B., and Agre, P. (1992) Science 256, 385-387
6. Zhang, R., Skach, W., Hasegawa, H., Van Hoek, A. N., and Verkman, A. S. (1992) J. Biol. Chem. 267, 18267-18269
7. Van Hoek, A. N., and Verkman, A. S. (1992) J. Biol. Chem. 267, 18267-18269
8. Zeidel, M. L., Ambudkar, S. V., Smith, B. L., and Agre, P. (1992) Biochemistry 31, 7436-7440
9. Sabolic, I., Valenti, G., Verbavatz, J. M., Van Hoek, A. N., Verkman, A. S., Ausiello, D. A., and Brown, D. (1992) Am. J. Physiol. 263, C1225-C1233
10. Nielson, S., Smith, B. L., Christensen, E. I., Knepper, M. A., and Agre, P. (1993) J. Cell Biol. 120, 271-283
11. Hasegawa, H., Zhang, R., Dohrmann, A., and Verkman, A. S. (1993) Am. J. Physiol. 33, C237-C245
12. Meyfeld, M. M., and Verkman, A. S. (1987) J. Membr. Biol. 96, 107-119
13. Van Heeswijj, M. F. E., and Van Os, C. H. (1986) J. Membr. Biol. 92, 183-193
14. Ye, R., Shi, L.-B., Lence, W., and Verkman, A. S. (1989) J. Gen. Physiol. 93, 385-392
15. Verkman, A. S., Weyer, P., Brown, D., and Ausiello, D. A. (1989) J. Biol. Chem. 264, 20608-20613
16. Verkman, A. S. (1992) Annu. Rev. Physiol. 54, 97-108
17. Finkelstein, A. (1987) in Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes: Theory and Reality, Wiley and Sons, New York
18. Van Hoek, A. N., Hoon, M. L., Lutjens, L. H., De Jong, M. D., Dempster, J. A., and Van Os, C. H. (1991) J. Biol. Chem. 266, 16633-16635
19. Preston, B. M., Jung, J. S., Guggino, W. B., and Agre, P. (1993) J. Biol. Chem. 268, 17-20
20. Zhang, R., Van Hoek, A. N., Biwersi, J., and Verkman, A. S. (1995) Biochemistry 34, 2938-2941
21. Verbavatz, J. M., Brown, D., Sabolic, I., Valenti, G., Van Hoek, A. N., Ma, T., and Verkman, A. S. (1993) J. Cell Biol. in press.
22. Felgner, P. L., and Ringold, G. M. (1989) Nature 337, 387-388
23. Zen, K., Biwersi, J., Periasamy N., and Verkman, A. S. (1992) J. Cell Biol. 119, 99-110
24. Verkman, A. S. (1987) J. Bioenerg. Biomembr. 19, 481-493
25. Zhang, R., and Verkman, A. S. (1990) J. Membr. Biol. 115, 253-261
26. Verkman, A. S., Lence, W., Brown, D., and Ausiello, D. A. (1989) Nature 333, 288-289
27. Shi, L.-B., Brown, D., and Verkman, A. S. (1995) J. Gen. Physiol. 95, 941-950
28. Balch, W. E., Dunphy, W. G., Breall, W. A., and Rothman, J. E. (1984) J. Cell Biol. 99, 405-416
29. Wikstrom, L., and Lodish, H. F. (1991) J. Cell Biol. 113, 997-1007
30. Tuli, R. S., Phope, D. J., and Tower, O. (1977) J. Biol. Chem. 252, 1723-1727
31. Deutscher, M. P. (1990) Methods Enzymol. 182, 203-225
32. Denker, B. M., Smith, B. L., Kuhajda, F. P., and Agre, P. (1988) J. Biol. Chem. 263, 15634-15642
33. Shi, L.-B., and Verkman, A. S. (1989) J. Gen. Physiol. 94, 1101-1116
34. Lucy, J. A. (1989) Biochim. Biophys. Acta 100, 623-624
35. Ebelerovic, M. V., and Verkman, A. S. (1992) J. Membr. Biol. 118, 253-261
36. Maci, R. (1984) Am. J. Physiol. 246, C105-C203
37. Rodman, J. S., Seidman, L., and Farquhar, M. G. (1986) J. Cell Biol. 102, 77-87
38. Verkman, A. S. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 11114-11115
39. Zeidel, M., Ambudkar, S. V., Smith, B. L., and Agre, P. (1992) Biochemistry 31, 7436-7440
40. Gregory, R. J., Cheng, S. H., Rich, D. P., Marshall, J., Paul, S., Hebner, K., Ostergaard, L., Klinger, K. W., Welsh, M. J., and Smith, A. E. (1990) Nature 347, 383-386
41. Scherer, T., Auld, V. J., Boyd, S., Offord, J., Dunn, R., and Catterall, W. A. (1990) Science 247, 534-537
42. Keyman, S., Suh, Y.-J., K. Kanne, B. L., and Rudnick, G. (1992) Biochemistry 31, 1974-1979
43. Lukacs, G. L., Chang, Y.-B., Karlin, N., Rotstein, O. D., Iordachita, J. R., and Grinstein, S. (1992) J. Biol. Chem. 267, 14588-14572
44. Kotliar, N., and Pilch, P. F. (1992) Mol. Endocrinol. 6, 337-345