Accelerated Publications

A Point Mutation at Cysteine 189 Blocks the Water Permeability of Rat Kidney Water Channel CHIP28k†

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ABSTRACT: CHIP28k is an important water-transporting protein in the kidney proximal tubule and the thin descending limb of Henle [Zhang, Skach, Hasegawa, Van Hoek, & Verkman (1993) *J. Cell Biol. 120*, 359–369] that is homologous to human erythrocyte CHIP28 [Preston & Agre (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11110–11114]. Oligonucleotide-directed mutagenesis was used to identify the cysteine(s) involved in inhibition of the water-transporting function of CHIP28k by the mercurial $HgCl_2$. Each of the four cysteines (at positions 87, 102, 152, and 189) were mutated to serine individually, or in combinations. In vitro transcribed cRNA was expressed in *Xenopus* oocytes for measurement of osmotic water permeability (P_f) in the absence or presence of 0.3 mM $HgCl_2$. P_f (in cm/s × 10^{-4} measured at 10 °C) was 7 ± 1 in water-injected oocytes. In wild-type CHIP28k, P_f was 58 ± 7 ($-HgCl_2$) and 12 ± 1 ($+HgCl_2$). Mutation of cysteine 87, 102, or 152, individually or in combinations, had little effect on oocyte P_f or on the inhibition by $HgCl_2$. Mutation of cysteine 189 to serine or glycine gave similar P_f values of 49–56 ($-HgCl_2$); however, P_f was not inhibited up to 1 mM $HgCl_2$. Mutation of cysteine 189 to the larger amino acid tryptophan gave a low P_f of 9 ± 1; coexpression with wild-type CHIP28k indicated that the tryptophan mutation was not dominant negative. Mutation of the asparagine 42 and 205 glycosylation sites to threonine had little effect on P_f. P_f in intact erythrocytes, but not inside-out erythrocyte vesicles, was inhibited by a membrane-impermeable mercurial (pCMB-dextran). These results indicate that cysteine 189 is the site of action of $HgCl_2$ and may reside at the external face of the CHIP28k aqueous pore.

CHIP28 (*ch*annel-forming *i*ntegral membrane *p*rotein of 28 kDa) is an abundant membrane protein in human erythrocytes that was recently isolated and cloned (Smith & Agre, 1991; Preston & Agre, 1991). When expressed in *Xenopus* oocytes, CHIP28 (Preston et al., 1992) and a homologous protein from rat kidney (CHIP28k; Zhang et al., 1993) strongly increased water but not ion permeabilities. Proteoliposomes reconstituted with purified CHIP28 had very high water permeability that was weakly temperature dependent and inhibited by the mercurial sulfhydryl-reactive compound $HgCl_2$ (Van Hoek & Verkman, 1992; Zeidel et al., 1992). CHIP28 is a selective water transporter that excludes urea and protons in reconstituted proteoliposomes and in native membrane vesicles. In situ hybridization (Hasegawa et al., 1993) and immunolocalization (Sabolic et al., 1992) studies indicate that CHIP28k mRNA and protein are present in the kidney proximal tubule and the thin descending limb of Henle, where it plays an important physiological role in transepithelial reabsorption of water. Functional (Verkman et al., 1989; Ye et al., 1989) and immunochemical (Sabolic et al., 1992) studies indicate that the CHIP28k (or a closely related) protein is present on both plasma and intracellular membranes in the kidney proximal tubule. In addition, mRNA encoding CHIP28k has been found in alveolar and intestinal crypt epithelium, corneal endothelium, and choroid plexus (Hasegawa et al., 1993), suggesting a wide and selective distribution in tissues that transport water rapidly.

CHIP28 has ~40% amino acid homology with members of the MIP26 (*m*ajor *i*ntrinsic *p*rotein of lens fiber of 26 kDa) family of proteins which include small (25–28 kDa) proteins from plants and bacteria and larger (70–80 kDa) proteins from *Drosophila* and yeast (Pao et al., 1991; Preston & Agre, 1991). Hydropathy analysis suggests that these proteins contain multiple membrane-spanning α-helices. CHIP28 is a transmembrane glycoprotein that may contain six (or possibly four) membrane-spanning α-helical domains (Figure 1A) (Preston & Agre, 1991; Zhang et al., 1993); recent analysis of the CHIP28 structure by circular dichroism and Fourier transform infrared spectroscopy indicates ~40% α-helical content (Van Hoek et al., 1993), consistent with the proposed structure based on hydropathy analysis. CHIP28 and CHIP28k contain four cysteine residues, two of which (C87 and C189) are predicted to reside in polar domains and two of which (C102 and C152) are predicted to reside in hydrophobic domains (Figure 1A). Of the two possible sites for N-linked glycosylation (N42 and N205), probably only a fraction (25–50%) of CHIP28 monomers are glycosylated at N42 (Zhang et al., 1993). Biochemical (Smith & Agre, 1991) and morphological (Verbavatz et al., 1992) studies suggest that CHIP28 molecules are assembled as tetramers in the membrane; however, target analysis studies suggest that the functional water-transporting unit is monomeric (Van Hoek et al., 1991). The route for movement of water through

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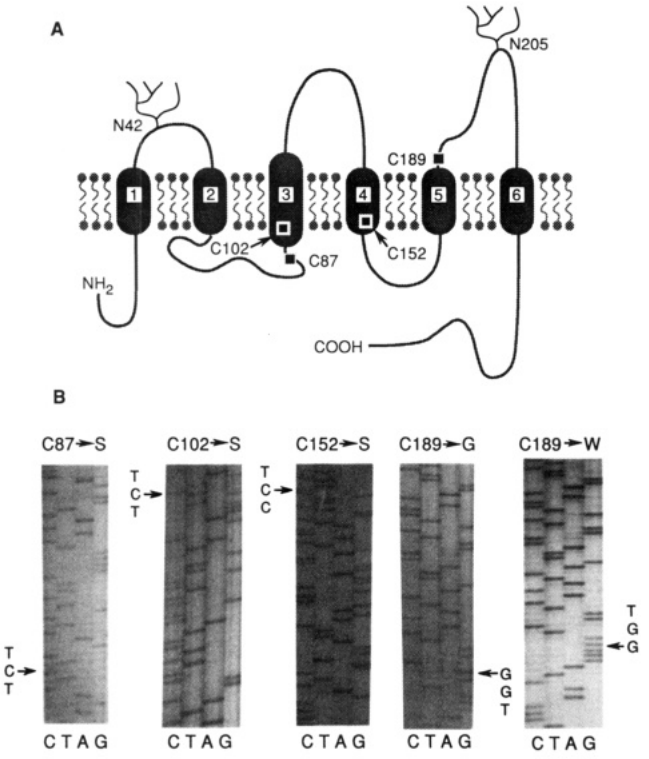

FIGURE 1: Proposed membrane topology of CHIP28k showing four cysteines (A) and representative sequencing gels showing single base mutations (B). The sequence (sense) is read from top (5′) to bottom (3′). Arrows indicate the mutated base.

the CHIP28 protein is not known, nor is the site of action of mercurial inhibitors.

The purpose of this study was to identify the cysteine(s) that are involved in the inhibition of CHIP28k function by the mercurial $HgCl_2$ and to determine whether glycosylation is important for function. The inhibition of water transport by $HgCl_2$ has been taken as a signature of proteinaceous water channels in a variety of tissues and subcellular fractions [for reviews, see Macey (1984), Harris et al. (1992), and Verkman (1992)]. In order to investigate the site(s) of action of $HgCl_2$, one or more cysteines in CHIP28k were mutated and the mutant proteins were expressed in *Xenopus* oocytes. It was found that mutation of cysteine 189 blocked the inhibitory effect of $HgCl_2$, whereas mutation of cysteines 87, 102, and 152, individually or in combinations, had no effect on CHIP28k water permeability or on the inhibition of water permeability by $HgCl_2$. Mutation of cysteine 189 to the larger amino acid tryptophan blocked water permeability. The cysteine 189 residue was localized to the external membrane surface by use of a novel membrane-impermeable mercurial inhibitor. The results suggest that cysteine 189 plays an important role in the CHIP28k aqueous pathway.

EXPERIMENTAL PROCEDURES

Oligonucleotide-Directed Mutagenesis. The altered sites in vitro mutagenesis system (Promega) was used to mutate the four cysteines of CHIP28k to serine or glycine by single base mutations. A *Hin*dIII–*Bam*HI fragment of plasmid PSP CHIP28k, containing the 807-bp coding sequence of CHIP28k (Zhang et al., 1993), was subcloned into the *Hin*dIII and *Bam*HI sites of plasmid pALTER-1 which has a functional tetracycline resistance gene and an inactivated ampicillin resistance gene. Transformed JM109 cells were grown on LB plates containing 15 μg/mL tetracycline and infected with helper phage M13K07 to produce the single-stranded template for mutagenesis.

Eight antisense mutagenic oligonucleotides (single mismatch underlined) were designed to change the four cysteines (C) of CHIP28k to serine (S), cysteine 189 to glycine (G) or tryptophan (W), and asparagines (N) 42 and 205 to threonine (T): (I) C87S, 5′ GCT GAT CTG AGA GCT GAG CAG 3′ (base +250 to +270); (II) C102S, 5′ GGC TCC CAC AGA CTG GGC GAT 3′ (base +295 to +315); (III) C152S, 5′ AGC CAG AAC GGA CAG CAC CAG 3′ (base +445 to +465); (IV) C189S, 5′ GTT GAT CCC AGA GCC AGT GTA 3′ (base +556 to +576); (V) C189G, 5′ GTT GAT CCC ACC GCC AGT GTA G 3′ (base +556 to +576); (VI) C189W, 5′ GG GTT GAT CCC CCA GCC AGT GTA 3′ (base +555 to +576); (VII) N42T, 5′ CAG CGT CTG GGT TCT CTC CAG 3′ (base +115 to +135); (VIII) N205T, 5′ GTT TGA GAA GGT GCG GGT GAG 3′ (base +604 to +624).

Oligonucleotides were phosphorylated by T_4 polynucleotide kinase. A 27-base phosphorylated ampicillin-repair oligonucleotide and individual or combinations of oligonucleotides were annealed to the ssDNA template at the ratio of 25:5:1 (mutagenic oligonucleotide:repair oligonucleotide:template). The heteroduplex containing the mutated strand was synthesized with T_4 DNA polymerase and transformed into the repair minus *Escherichia coli* BMH 71–18 mutS. Mutants were selected by overnight growth in LB medium containing 125 μg/mL ampicillin. Plasmid DNA was then isolated and transformed into JM109-competent cells for growth on LB plates containing ampicillin. Mutants were screened by direct sequencing of plasmid DNA isolated from ampicillin-resistant colonies. Representative sequencing gels showing mutated cDNAs are given in Figure 1B. The sequence was obtained by the dideoxynucleotide method using a sequenase kit (Version 2, USB).

In Vitro Transcription and Oocyte Expression. Plasmid pALTER-1 (containing mutated DNAs) was prepared by the Magic Miniprep DNA purification system (Promega). The plasmid was linearized with *Sca*I and transcribed/capped with SP6 RNA polymerase using the mCAP mRNA capping kit (Stratagene). Stage V and VI oocytes from *Xenopus* laevis were isolated, defolliculated with collagenase (type 1A, Sigma, 2 mg/mL, 2 h, 20 °C), and maintained in Barth's buffer (200 mOsm). Oocytes were microinjected with 50-nL samples of mRNA (0–1 mg/mL) as described (Zhang et al., 1990, 1991) and incubated at 18 °C for 40–48 h.

Osmotic water permeability (P_f) in oocytes was measured by a swelling assay (Zhang & 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 time intervals by a quantitative imaging method. Temperature was maintained at 10 °C by a circulating water bath. Oocyte P_f was calculated from the initial rate of swelling, $d(V/V_0)/dt$, by the relation $P_f = [d(V/V_0)/dt]/[(S/V_0)V_w(Osm_{out} - Osm_{in})]$, where $S/V_0 = 50\ cm^{-1}$, $V_w = 18\ cm^3/mol$, and $Osm_{out} - Osm_{in} = 190$ mOsm. In some experiments, 0–1 mM $HgCl_2$ was present in the diluted extracellular buffer. In some experiments, oocytes were incubated for 5 min with 0.3 mM $HgCl_2$ and then for 5 min with 5 mM β-mercaptoethanol prior to water transport assay.

Inhibition of Erythrocyte Water Permeability by a Membrane-Impermeable Mercurial. p-(Chloromercuri)benzoate (pCMB) was conjugated to dextran (10 kDa) by reaction of 100 mg of aminodextran, 30 mg (0.084 mmol) of pCMB, and 32 mg (0.168 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride in 10 mL of 0.1 M $NaHCO_3$ (pH ~ 8.3) for 8 h in the dark. The reaction mixture was

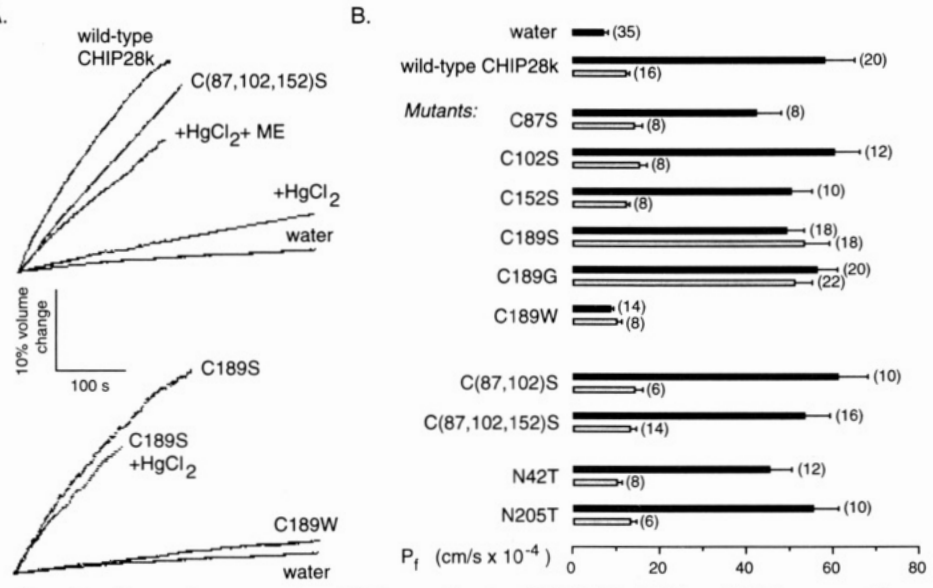

FIGURE 2: Time course of swelling for oocytes expressing wild-type and mutant CHIP28k cRNAs. (A) Representative experiments on single oocytes. Oocytes were injected with 50 μL of water or in vitro transcribed cRNA (0.1 mg/mL) encoding wild-type CHIP28k or the indicated mutants. Osmotic water permeability was assayed at 10 °C at 40–48 h after injection from oocyte swelling in response to a 20-fold dilution of Barth's buffer with distilled water. Where indicated +$HgCl_2$, the assay solution contained 0.3 mM $HgCl_2$. Where indicated +$HgCl_2$ + ME, 5 mM β-mercaptoethanol was added after incubation with $HgCl_2$ (see Experimental Procedures). (B) Summary of oocyte P_f values (mean ± SEM) with the number of oocytes in each group shown in parentheses. The light bar indicates 0.3 mM $HgCl_2$. The cysteine-to-serine combination mutants are denoted C(87,102)S and C(87,102,152)S.

dialyzed for >36 h until unconjugated pCMB was absent and then lyophilized. Using molar absorbance data, the pCMB-to-dextran molar labeling ratio was 1:4.

Osmotic water permeability in human erythrocytes and inside-out vesicles (IOVs) derived from human erythrocytes was measured by the stopped-flow light scattering method (Van Hoek & Verkman, 1992) at 10 °C. The pCMB-dextran inhibitor was incubated with membranes for 30 min prior to assay of water transport.

RESULTS AND DISCUSSION

To determine which of the four cysteines in CHIP28k are involved in water transport and in the inhibition of water transport by $HgCl_2$, we first mutated individual cysteines to serine by single base changes. Mutations were confirmed by sequence analysis, and the cDNAs were in vitro transcribed to produce cRNA for expression in *Xenopus* oocytes. Figure 2A shows representative data for the time course of oocyte swelling after a 20-fold dilution of the extracellular Barth's buffer with distilled water; the initial slope provides a quantitative measure of osmotic water permeability (P_f). Figure 2B gives the mean and SEM for a series of measurements performed on individual oocytes. In all studies, P_f was measured at 10 °C (to minimize the contribution of water movement through lipids; Zhang & Verkman, 1991) and in the absence and presence of 0.3 mM $HgCl_2$, a concentration found to inhibit P_f in oocytes expressing wild-type CHIP28k (Zhang et al., 1993).

The averaged data in Figure 2B show that cysteine-to-serine mutations of cysteines 87, 102, 152, and 189 had little effect on oocyte water permeability in the absence of $HgCl_2$. $HgCl_2$ effectively inhibited P_f induced by the C87S, C102S, and C152S mutants; however, little or no inhibition by $HgCl_2$ was observed for the C189S mutant, suggesting that cysteine 189 is the site of $HgCl_2$ inhibition. The curves in Figure 2A (top) and the averaged data in Figure 2B indicate no significant effect of mutation of cysteines 87, 102, and 152 (together) on P_f or on the inhibition of P_f by 0.3 mM $HgCl_2$. The inhibition of P_f by $HgCl_2$ in the (C87, C102, C152) combination mutant was reversed by ME [$P_f = (45 \pm 5) \times 10^{-4}$ cm/s, $n = 6$], similar to results obtained with the wild-type CHIP28k. The ability of $HgCl_2$ to inhibit P_f fully in the (C87, C102, C152) combination mutant supports the conclusion that cysteine 189 is the site of $HgCl_2$ inhibition.

To investigate whether the loss of water transport inhibition by $HgCl_2$ was a unique finding for the cysteine-to-serine mutation, cysteine 189 was mutated to glycine (C189G) and tryptophan (C189W). Figure 2B shows that the C189G mutant gave a P_f similar to that of the C189S mutant, with no inhibition by $HgCl_2$. In separate studies, 1 mM $HgCl_2$ did not affect P_f in oocytes expressing the C189S or C189G mutant proteins. Mutation of cysteine 189 to the larger amino acid tryptophan was associated with low P_f even in the absence of $HgCl_2$. The decreased P_f observed for the C189W mutant may be due to occlusion of the aqueous pore by its larger size or to abnormal processing resulting in altered topology and/or cellular targeting. These data indicate that cysteine 189 is the site of action of $HgCl_2$ and is probably a critical residue for the water-transporting activity of CHIP28k. Figure 2B also shows that mutation of the putative glycosylation sites (asparagines 42 and 205) to threonine (N42T and N205T mutants) had little effect on P_f or on the inhibition of P_f by $HgCl_2$.

To investigate whether functional water permeability required an oligomeric assembly of CHIP28k monomers, P_f was measured in oocytes coexpressing wild-type and mutant CHIP28k. Table I indicates that P_f in oocytes injected with 1 ng of cRNA encoding wild-type CHIP28k was not affected by coinjection of up to 10 ng of cRNA encoding the C189W mutant. Thus, if heterooligomers containing wild-type and C189W mutant proteins are formed, then the functional unit of CHIP28k may be the monomer. However, these experiments do not exclude the possibility that wild-type homo-oligomers are formed even in the presence of the mutant protein. Therefore, studies were carried out in oocytes coexpressing the functional wild-type and C189G proteins. Table I shows that, with increasing amounts of injected cRNA encoding the C189G protein, P_f increased, and the inhibitory

Table I: Coexpression of Wild-Type and Mutant CHIP28k in Oocytes[a]

amt of cRNA injected (ng)			P_f (cm/s × 10^{-4})	
wild type	C189G	C189W	$-HgCl_2$	$+HgCl_2$
1	0	0	32 ± 2	9 ± 1
1	0	1	35 ± 3	9 ± 1
1	0	5	29 ± 4	10 ± 1
1	0	10	29 ± 4	9 ± 2
0	0	10	9 ± 1	10 ± 1
1	1	0	41 ± 3	27 ± 2
1	5	0	61 ± 7	50 ± 4
1	10	0	70 ± 8	63 ± 6

[a] P_f was measured at 10 °C in oocytes injected with the indicated nanogram quantities of cRNA encoding wild-type and/or mutant CHIP28k proteins. Data are given as mean ± SEM for 10–18 oocytes in each group. Inhibition was measured in the presence of 0.3 mM $HgCl_2$.

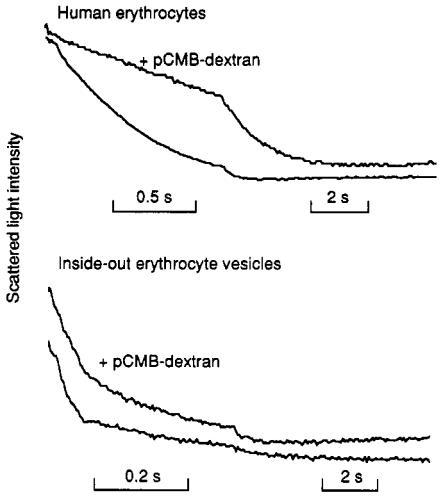

FIGURE 3: Inhibition of water permeability in erythrocytes. Intact human erythrocytes or inside-out erythrocyte membrane vesicles in phosphate-buffered saline were subjected to a 300 mM sucrose gradient in a stopped-flow apparatus at 10 °C. Scattered light intensity increases as vesicles shrink. Where indicated, 50 mg/mL pCMB-dextran was incubated with membranes for 30 min prior to measurement.

potency of $HgCl_2$ decreased. These results are consistent with the possibility that monomeric CHIP28k units are acting independently and are functional.

The structural prediction for CHIP28k in Figure 1A places cysteine 189 in an external extramembrane domain. To test this prediction, we synthesized a membrane-impermeable mercurial compound, pCMB-dextran. Figure 3 shows that pCMB-dextran inhibited osmotic water permeability in intact erythrocytes but not in inside-out erythrocyte membrane vesicles. In control experiments performed in intact erythrocytes, equivalent concentrations of (unconjugated) dextran did not inhibit water permeability, and the inhibition of water permeability by pCMB-dextran was reversed by ME. The results indicate that cysteine 189 is accessible only from the external membrane side.

We conclude from these findings that cysteine 189 is important for the water-transporting function of the CHIP28k water channel. The decreased water permeability observed when cysteine 189 was mutated to the larger amino acid tryptophan suggests that cysteine 189 resides in the aqueous pore; the lack of a dominant negative effect of the tryptophan mutation is consistent with (but does not prove) that the functional unit of CHIP28k is the monomer. The lack of $HgCl_2$ inhibition for the cysteine 189 mutants, and the finding that mutation of the other three cysteines had no effect on P_f or on the inhibition of P_f by $HgCl_2$, indicates that cysteine 189 is the site of action of mercurial sulfhydryl transport inhibitors. The ability of a membrane-impermeable mercurial compound to block P_f in erythrocytes but not in inside-out erythrocyte membranes indicates that cysteine 189 faces the outer membrane surface. These studies do not provide information about whether mutation of cysteine 189 causes changes in CHIP28k topology and/or the multimeric assembly of CHIP28k monomers in the membrane.

Amino acid sequence alignment of CHIP28k with members of the MIP family of proteins (including NOD, MIP, BiB, TIP, TUR, and GLP) reveals a number of common features, including a tandem internal repeat with a highly conserved region called the NPA box, and several conserved glycine residues (Pao et al., 1991; Wistow et al., 1991). Sequence alignment does not predict a highly conserved cysteine residue; however, when CHIP28k, NOD, MIP, and BiB are aligned, a conserved cysteine can be assigned, corresponding to cysteine 152 of CHIP28k. In the experiments reported here, mutation of cysteine 152 was without effect. At this time it is not known whether members of the MIP family are water transporters and whether water transport is inhibited by mercurials.

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