Molecular Cloning of a Mercurial-insensitive Water Channel Expressed in Selected Water-transporting Tissues*

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Two mercurial-inhibitable water-transporting proteins have been identified: CHIP28, an erythrocyte water channel also expressed in kidney tubules and selected extrarenal epithelia, and WCH-CD, a kidney collecting duct water channel. In searching for a protein responsible for the high transcellular water permeability in lung alveolus, we cloned a 32-kDa water channel (mercurial-insensitive water channel (MIWC)) from a rat lung cDNA library with several novel features. Water permeability was strongly increased in Xenopus oocytes expressing MIWC in a mercurial-insensitive manner, in contrast to known water channels. By in situ hybridization, MIWC showed an unique distribution in cells that do not express CHIP28, including kidney papillary vasa recta, cells lining the subarachnoid space and ventricles in brain, the inner nuclear layer in retina, and the conjunctival epithelium. An alternatively spliced form of MIWC with a 165-base pair deletion in the coding sequence was also identified; relative expression of the spliced mRNA was tissue-specific. The MIWC water channel may participate in the urinary concentrating mechanism, the absorption of cerebrospinal fluid, and other physiological processes.

Recent studies indicate that two members of the MIP (Major Intrinsic Protein of lens fiber) protein family function as physiologically important water channels in mammals. CHIP28 is a major water-transporting protein in erythrocytes, kidney proximal tubule, and thin descending limb of Henle (1–5), and epithelial cells in choroid plexus, ciliary body, alveolus, intestinal crypt, and other tissues (6, 7). CHIP28 is a mercurial-inhibited water-transporting protein (8) that does not pass urea, protons, or monovalent ions (9–12). A second mercurial-sensitive water channel expressed exclusively in mammalian kidney collecting duct (WCH-CD) was identified recently (13, 14).

There are a number of water-permeable plasma membranes in fluid-transporting mammalian tissues that do not express known water channels. Based on functional studies showing high transcellular water permeability in intact lung alveolus (15), the purpose of our study was to identify and clone a second lung water channel, which together with CHIP28 could confer high transcellular water permeability in lung. A novel mercurial-insensitive water channel (MIWC) reported here was cloned from rat lung but was also expressed strongly in kidney inner medulla, in cells involved in the reabsorption of cerebrospinal fluid in brain, and in other tissues.

EXPERIMENTAL PROCEDURES
cDNA Cloning of MIWC—Degenerate oligonucleotide primers were designed against conserved motifs in MIP26 family members (14); sense, 5′-CACTTTTGAATACAACACCTGCCGCTGAGCTGACTGTTGAGGUAGCAGATTCTGCAGTTT-3′; antisense, 5′-CCTGCAGCAGTTTCTTCTTTTCTTCTTCAGTTTCCATCGTTCT-3′. PCR amplification was performed using 100 pmol of degenerate primers with reverse transcribed rat lung cDNA as template (1 min at 94 °C, 1 min at 59 °C, 2 min at 72 °C, 30 cycles). A band of ~400 bp was isolated by agarose gel electrophoresis and subcloned into TA cloning vector pCRIII (Invitrogen). Inserts were sequenced by the double-strandideoxy-nucleotide termination method (U. S. Biochemical Corp. kit). –10× plagues from a rat lung 5′-stretch -1 kb cDNA library (Clontech) were screened under high stringency (hybridization in 6 × SSC, 6 × Denhardt’s solution, 1% SDS, 10 μg/ml Tris-HCl, 0.1% dextran sulfate at 58 °C for 20 h and washing in 2 × SSC, 0.1% SDS at 23 °C for 30 min). Two positive clones with –1.2- and –1.3-kb insert sizes were subcloned into plasmid vector pGEM-3Zf(+) (Promega) at EcoRI sites and sequenced. The 1.2-kb cDNA contained the full-length coding sequence for functional MIWC; the 1.3-kb cDNA was identical in overlapping regions, except that the coding sequence contained a 165-bp deletion (see below).

Northern and Southern Blot Analysis—Poly(A) + RNA from rat lung and kidney were prepared by phenol/chloroform extraction and oligo(dT) affinity chromatography. Poly(A) + RNAs (6 μg/lane) were used as template for PCR amplification with sense primer A, 5′-GCTGATCATGGTGGCCCTAAAGGCGTCTGC-3′, and antisense primer B, 5′-CCGGCATGACTTGGTGCTTACAAGGGGCTCTC-3′, corresponding to bp –3 to +876 of MIWC with a 5′-SpI site. PCR products were resolved on a 1% agarose gel and transferred to a nitrocellulose filter. A radiolabeled MIWC probe (bp –3 to +876) was prepared by random priming. Hybridization was performed at 42 °C for 18 h with 100 μg/ml denatured salmon sperm DNA in 2 × Denhardt’s solution, 2% SDS, 5 × SSC, 50% deionized formamide. RNAs were isolated from rat brain, eye, trachea, lung, heart, kidney, stomach, colon, liver, and salivary gland. Reverse transcribed cDNAs were used as template for PCR amplification with sense primer A, 5′-GCTGATCATGGTGGTGCTTACAAGGGGCTCTC-3′, corresponding to bp –3 to +23 of MIWC with an engineered 5′-BcI site, and antisense primer B, 5′-CCGGCATGACTTGGTGCTTACAAGGGGCTCTC-3′, corresponding to bp +889 to +876 of MIWC with a 5′-SpI site. PCR products were resolved on a 1% agarose gel and transferred to a nitrocellulose filter. Hybridization was performed under high stringency (6 × SSC, 6 × Denhardt’s solution, 1% SDS, 10 μg/ml Tris-HCl) with MIWC probe at 58 °C overnight.

Expression of MIWC and mMIWC in Xenopus Oocytes—The coding sequence of MIWC was amplified by PCR using sense primer A and antisense primer corresponding to SpI promoter in pGEM-32zf(+). The PCR product (corresponding to bp –3 to +1120 of MIWC) was subcloned into pSP64T (16) at BclI/BglII, and EcoRI sites. The resultant plasmid pSP-MIWC contained the Xenopus β-globin 5′-untranslated region located 11 bp upstream of the MIWC ATG initiation codon. cRNA encoding MIWC was transcribed/capped using an in vitro transcription kit (Stratagene). Stage V and VI oocytes from Xenopus laevis were defolliculated and microinjected with 10–30 nl of cRNA encoding MIWC
**MIWC Cloning**

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**A**

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**B**

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**C**

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**Fig. 1. Nucleic acid and deduced amino acid sequence of MIWC.** A, symbols (*, *, and **) indicate consensus sites for N-linked glycosylation, and protein kinase A and C phosphorylation, respectively. The first ATG was identified as the translation initiation codon by Kozak's consensus sequence. The box indicates the absent region in spliced sMIWC. B, deduced amino acid sequence aligned to water channels CHIP28k and WCH-CD. C, Kyte-Doolittle hydrophobicity profile of deduced amino acid sequence of MIWC.
Fig. 2. Expression and tissue distribution of MIWC. A, Northern blot analysis of MIWC expression in lung and kidney. Lung and kidney poly(A)+ RNAs (10 μg/lane) were subjected to Northern blot analysis with MIWC probe. B, Southern blot analysis of PCR products. Reverse transcribed cDNAs (450 ng/lane) from indicated tissues were subjected to PCR amplification and analyzed by Southern blot (see "Experimental Procedures"). Symbols (arrowhead) and (double arrowhead) represent bands corresponding to MIWC and sMIWC, respectively.

(−0.2 μg/ml). After a 24-h incubation in Barth’s buffer at 18 °C, osmotic water permeability (PΩ) was measured from the time course of oocyte swelling in response to a 3-fold dilution of Barth’s buffer with distilled water (12). Arrhenius activation energy (Ea) was calculated from a plot of ln PΩ versus reciprocal absolute temperature. (14)Urea, (3H)Sucrose, and, ion permeabilities were measured as described previously (16).

Cell-free Translation—In vitro transcribed cRNA was added to a rabbit reticulocyte lysate mixture containing [35S]methionine (12). Translation was performed at 24 °C for 1 h. Microsomal membranes prepared from dog pancreas were added at the start of translation to a final concentration of 8 A260. An acceptor peptide (AcAsn-Tyr-Thr) was added to determine whether glycosylation occurred.

In Situ Hybridization—A 170-bp SacII/SphI fragment corresponding to bp +707 to +876 of MIWC was subcloned into pGEM-3Zf+(). Sense and antisense cRNA probes were transcribed with T7 and SP6 RNA polymerase in the presence of [35S]UTP (10 μCi/ml) after plasmid linearization with HindIII and EcoRI, respectively. Hybridization was carried out as described previously (6).

RESULTS AND DISCUSSION

To identify putative lung water channels, sequences containing two amino acid motifs (NPA boxes) that are conserved between kidney and lung (Fig. 2A). Interestingly, part of the “Bib” DNA sequence (bp +463 to +1350, Ref. 18) and a 388-bp human brain expressed gene tag “EST02306” (19), which was obtained by sequencing 2,375 human brain cDNAs, showed 51 and 86% DNA sequence identity to MIWC, respectively. The homology suggests that MIWC is closely related to the Bib protein whose function has not been defined.

Northern blot analysis showed a hybridization at −5.5 kb in both kidney and lung (Fig. 2A). The tissue distribution of mRNA encoding MIWC was studied by Southern blot analysis of PCR fragments produced by amplification of reverse transcribed cDNAs from a series of tissues using MIWC-specific primers. Hybridization was observed at −0.9 kb (corresponding to the MIWC probed region, bp −3 to +876) in brain, eye, lung, kidney, colon, liver, and salivary gland (Fig. 2B). Interestingly, a smaller PCR product of −0.8 kb hybridized in all positive lanes. The relative intensity of the two bands was tissue-specific, with significantly more of the smaller band in liver and salivary gland. From sequence analysis, the smaller PCR product was identical to sMIWC. The deleted region (amino acids 128–182) is nearly equivalent to exon 2 of human CHIP28 genomic DNA (corresponding to amino acids 129–183 of human CHIP28, Ref. 20), suggesting that sMIWC is an alternatively spliced form of MIWC expressed in a tissue-specific manner. Other minor bands were detected in brain, eye, and lung (Fig. 2B), which may represent other spliced forms.

Osmotic water permeability (PΩ) was measured in Xenopus oocytes injected with transcribed cRNA encoding MIWC, sMIWC, and CHIP28k (Fig. 3A). PΩ in MIWC-injected oocytes was 51 ± 9 × 10−4 cm/s (S.E., n = 15), which was more than 10 times greater than that in water-injected oocytes (5 ± 2 × 10−4 cm/s, n = 6). In contrast, sMIWC did not function as a water channel (PΩ = 5 ± 1 × 10−4 cm/s, n = 9). Temperature dependence measurements gave an activation energy for PΩ in MIWC-injected oocytes of 3.6 kcal/mol, supporting the existence of an aqueous pore (17). Interestingly, PΩ in MIWC-injected oocytes
was inhibited little by HgCl2 (16 ± 11% inhibition, 0.3 mm HgCl2, n = 9; 24 ± 12%, 0.7 mm HgCl2, n = 5), whereas PT in CHIP28k-injected oocytes was strongly inhibited (88 ± 6%, 0.3 mm HgCl2, n = 10 (Fig. 3B)). The insensitivity of MIWC water permeability to HgCl2 is consistent with the presence of alanine at three amino acids upstream from the second NPA box (position 188) instead of cysteine (position 189 in CHIP28 and position 181 in WCH-CD), which has been shown to be the site of HgCl2 action in known water channels (8). The weak inhibition of MIWC water permeability at 0.7 mm HgCl2 may represent a toxic effect of HgCl2.

To determine MIWC transport selectivity, measurements of solute and ion permeability were made. [14C]Urea permeability in water-injected oocytes (P urea = 3.3 ± 0.8 × 10⁻⁶ cm/s, n = 6, 23 °C) was not different from that in MIWC-expressing oocytes (3.8 ± 1.2 × 10⁻⁶ cm/s, n = 6). Both types of oocytes were impermeable to [14C]Sucrose. Ion permeability, measured by two-electrode voltage clamp, also did not differ (range, 1-3 mV cm/s). Therefore MIWC is a selective transporter for water. Cell-free translation of MIWC in rabbit reticulocyte lysate supplemented with pancreatic microsomes produced a single protein band migrating at ~30 kDa (Fig. 3C). Addition of acceptor peptide did not alter the translated products, indicating that MIWC was not glycosylated.

In situ hybridization revealed a unique tissue distribution of mRNA encoding MIWC. In the kidney, hybridization with antisense probe was observed only in inner medulla (Fig. 4, A and B); analysis of alternate sections probed with antisense CHIP28 and WCH-CD cRNA showed that MIWC was not expressed in proximal tubule, thin descending limb of Henle, and collecting duct, suggesting that MIWC expression was localized to vasa recta or ascending limb of Henle. This hybridization sharply contrasts with that of CHIP28 in proximal tubule and thin descending limb of Henle (1, 2) and of WCH-CD in cortical and medullary collecting duct (13, 14). Based on a perfusion study of descending vasa recta (21), calculated P in descending vasa recta is 0.027 cm/s, similar to P in erythrocytes. In brain, MIWC mRNA was expressed strongly in the cells lining the ventricular surface (Fig. 4, C and D) and cells covering brain matter in contact with the subarachnoid space (Fig. 4, E and F), while it was absent in choroid plexus that strongly expresses CHIP28 mRNA (6). Cerebrospinal fluid is probably secreted through the CHIP28 water channel and absorbed through the MIWC. In eye, MIWC was expressed in the inner nuclear layer of retina (Fig. 4, G and H) and the conjunctival epithelium, which is known to produce mucin for cornea protection; MIWC was not expressed in corneal endothelium and epithelium of ciliary body, iris, and lens surface in which CHIP28 is expressed (2, 6, 7). In lung, MIWC was expressed diffusely in alveolus with a distribution similar to that of CHIP28 mRNA (2). No selective hybridization was observed with MIWC sense probe in any tissue.

MIWC is the first mercurial-insensitive water channel identified, indicating that HgCl2 inhibition is not a conserved feature of cellular water channels. The alternative splicing of MIWC may play a role in the tissue-specific regulation of water permeability. The unique tissue distribution of mRNA encoding MIWC suggests physiological involvement in the urinary concentrating mechanism, the reabsorption of cerebrospinal fluid, the resolution of alveolar edema, and other processes.

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