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Water Channel Properties of Major Intrinsic Protein of Lens*

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The functions of major intrinsic protein (MIP) of lens are still unresolved; however the sequence homology with channel-forming integral membrane protein (CHIP) and other Aquaporins suggests that MIP is a water channel. Immunolocalizations confirmed that XeRosenberg, 1985; Girsch and Peracchia, 1985; Peracchia and Girsch, 1989; Shen et al., 1991). In planar lipid bilayers, MIP forms single channels of various conductances (up to 3 nanosiemens) and a weak selectivity for anions (Ehring et al., 1990); Nodulin-26, a homologous protein from soy bean root nodules, was recently shown to behave similarly (Weaver et al., 1994). In spite of these studies with reconstituted MIP protein, studies of MIP in native membranes or expressed in oocytes have failed to confirm a physiologic function for MIP. For example, although MIP was initially considered to be the gapjunction protein, immunological, biochemical, and electrophysiological studies failed to identify electric coupling of lens fiber cells via MIP (Swenson et al., 1989). MIP was the first identified member of an ancient family of membrane proteins from diverse organisms that now includes more than 20 members. Several MIP family members from animal and plant tissues were shown to function as waterselective membrane channels and are now referred to as the "Aquaporins" (Chrispeels and Agre, 1994). The purification and cDNA cloning of the 28-kDa channel-forming integral membrane protein, CHIP (Denker et αl ., 1988; Preston and Agre, 1991), a MIP homolog from red cells and renal proximal tubules, permitted the first demonstration of a molecular water channel (Preston et al., 1992). CHIP is now designated Aquaporin-1 (AQP1), and cDNAs encoding four other Aquaporins have subsequently been isolated from diverse mammalian tissues (Fushimi et al., 1993; Ishibashi et al., 1994; Ma et al., 1994; Echevarria et al., 1994; Hasegawa et al., 1994; Jung et al., 1994b; Raina et al., 1995). The hourglass model was recently proposed to merge structural and functional features of the Aquaporins (Jung et al., 1994a). The model describes two tandem repeats with the first and second half of the molecule oriented at 180° to each other, and each half contains the sequence asparagine-proline-alanine (NPA) in intracellular loop B and extracellular loop E. In the hourglass model, it was predicted that loop B and loop E fold back into the membrane, forming a single water-selective pore. The striking sequence homology with the Aquaporins suggests that MIP may also be involved in water movement through the lens fiber cell membranes. In this report, we investigated the water transporting capacities of MIP using the *Xenopus* oocyte expression system, and water channel properties qualitatively similar to the Aquaporins were observed.

nopus oocytes injected with bovine MIP cRNA express the protein and target it to the plasma membrane. Control oocytes or oocytes expressing MIP or CHIP exhibited small, equivalent membrane currents that could be reversibly increased by osmotic swelling. When compared with water-injected control oocytes, the coefficient of osmotic water permeability (P_f) of MIP oocytes was increased 4-5-fold with a low Arrhenius activation energy, while the P_f of CHIP oocytes increased > 30-fold. To identify structures responsible for these differences in P_p recombinant MIP proteins were expressed. Analysis of MIP-CHIP chimeric proteins revealed that the 4-kDa cytoplasmic domain of MIP did not behave as a negative regulator. Individual residues in MIP were replaced by residues conserved among the Aquaporins, and introduction of a proline in the 5th transmembrane domain of MIP raised the P_f by 50%. Thus oocytes expressing MIP failed to exhibit ion channel activity and consistently exhibited water transport by a facilitated pathway that was qualitatively similar to the Aquaporins but of lesser magnitude. We conclude that MIP

functions as an Aquaporin in lens, but the protein may also have other essential functions.

Major intrinsic protein $(MIP)^1$ is a 26-kDa protein expressed exclusively in lens fiber cells where it comprises over 60% of the membrane protein. The cDNA encoding MIP was isolated from a bovine lens cDNA library, and hydrophobicity plots predicted that MIP is an integral membrane protein with cytoplasmic amino and carboxyl termini and six bilayer-spanning domains (Gorin *et al.*, 1984). MIP reconstituted into liposomes exhibits voltage-dependent channels permeable to ions and small molecules that may be closed by Ca²⁺ and calmodulin (Nikaido and

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** To whom correspondence may be addressed: Dept. of Cell Physiology, Trigon Bldg. Adelbertusplein 1, University of Nijmegen, 6500 HB Nijmegen, The Netherlands. Fax: 31 80 54 05 25. ‡‡ To whom correspondence may be addressed: Dept. of Biological Chemistry, Physiology Bldg., Rm. 420, Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205-2185. Fax: 410-955-3149. ¹ The abbreviations used are: MIP, major intrinsic protein of lens; P_f , coefficient of osmotic water permeability; CHIP, channel forming integral protein of 28-kDa (CHIP28).

EXPERIMENTAL PROCEDURES

Plasmid DNA Mutagenesis, DNA Sequencing, and in Vitro RNA Synthesis—Standard molecular procedures were used (Sambrook et al., 1989). The bovine MIP coding sequence (Gorin et al., 1984) flanked 5' and 3' by Xenopus β -globin gene untranslated sequences (Swenson et al., 1989) was transferred into pBluescript II KS (Stratagene) following digestion with HindIII and XbaI. The CHIP expression vector was constructed as described (Preston et al., 1992). These constructs served as templates for site-directed mutagenesis reactions using the Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad). Table I lists the

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Site-specifi	TABLE I c mutations, insertional mutations, and chimeric constructs of M	IP and CHIP
MIP constructs	Wild-type amino acid/codon	Mutant amino prid/redev
MIP (V112Bam) MIP(C14V) MIP(V160P) MIP(A181C) MIP(S243V/S245E)	Val-112/GTC Cys-14/TGT Val-160/GTG Ala-181/GCA Ser-Glu-Ser-245/AGTgagTCC	Val-Asp-Pro/GTggatccC Val-14/GTT Pro-160/CCA Cys-181/TGT Val-Glu-Glu/GTTgagGAA
CHIP constructs	Wild-type amino acid/codon	Mutant amino acid/codon
CHIP(T120Bam) CHIP(D131P) CHIP(P169A)	Thr-120/ACT Asp-131/GAT Pro-169/CCC	Thr-Asp-Pro/ACggatccT Pro-131/CCT Ala-169/GCC
Chimoric constructs	Amino acids	Total amino acids
MIP-CHIP (loop A) MIP-CHIP-1 MIP-CHIP-2 CHIP-MIP-1 CHIP-MIP-2	MIP(1-32)-CHIP(34-51)-MIP(44-263) MIP(1-112)-CHIP(121-269) MIP(1-226)-CHIP(235-269) CHIP(1-120)-MIP(113-263)	270 263 261 273

CHIP(1-234)-MIP(227-263)

mutants used in this study. The locations of mutations within the MIP polypeptide are illustrated in Fig. 1.

The chimeric protein vectors CHIP-MIP-1 and MIP-CHIP-1 were constructed by inserting a BamHI restriction site at Val-112 of MIP and Thr-120 of CHIP, resulting in the insertion of the amino acids aspartic acid and proline. MIP(V112Bam) and CHIP(T120Bam) were digested with BamHI, the 5'-half of CHIP was ligated to the 3'-half of MIP (CHIP-MIP-1), and the 5'-half of MIP was ligated to the 3'-half of CHIP (MIP-CHIP-1). The chimeric protein vectors MIP-CHIP-2 and CHIP-MIP-2 were constructed using the megaprimer polymerase chain reaction method (Barik and Galinski, 1991) exchanging at Arg-226 of MIP and Arg-234 of CHIP, using the following antisense primers: MIP-CHIP-2. 5'-GTCTGTGAGGTCACTGCTG-CGAGGGAAGAGGAGA-AAG-3'; CHIP-MIP-2, 5'-CTCAGAAACACTCTTGAGC-CGTGGGGC-CAGGATGAAG-3'.

The chimeric protein, MIP-CHIP (loop A), contains the first exofacial loop (A) of CHIP (amino acids 34-51) in place of the first exofacial loop of MIP (amino acids 33-43) and was constructed with an 82-base pair insertional-substitutional oligonucleotide primer (not shown) in a sitedirected mutagenesis reaction. All mutations were confirmed by enzymatic nucleotide sequencing (U.S. Biochemical Corp.).

Capped RNA transcripts were synthesized in vitro using T3 RNA



FIG. 1. Membrane topology of MIP showing sites targeted for

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polymerase with Xbal-digested MIP, CHIP, or mutant expression vector DNA, and the RNA was purified as described (Yisraeli and Melton, 1989).

Preparation of Occytes and Measurement of Pf-Female Xenopus *lacuis* were anesthetized on ice, and stage V and VI occytes were removed and prepared (Lu et al., 1990). The day after isolation, occytes were injected with either 50 nl of water or 0.5–25 ng of cRNA in 50 nl of water. Injected occytes were maintained for 2-3 days at 18 °C prior to osmotic swelling, membrane isolation, or voltage clamp experiments. Ocyte swelling was performed at 22 °C following transfer from 200 moss (osmin) to 70 moss (osminit, CHIP) or either 70 or 20 moss (osminite MIP) modified Barth's solution diluted with water. Sequential oocyte images were digitized at 5-s intervals for a total of 1 min, and the volumes of the sequential images were calculated as described (Preston et al., 1993). The change in relative volume with time, $d(V/V_0)/dt$, was fitted by computer to a quadratic polynomal, and the initial rates of swelling were calculated. The osmotic water permeability ($P_{\rho} \mu m/s$) was calculated from osmotic swelling data between 5 and 10 s, initial oocyte volume ($V_0 = 9 \times 10^{-4} \text{ cm}^3$), initial surface area ($S = 0.045 \text{ cm}^2$), and the molar ratio of water ($V_w = 18 \text{ cm}^3/\text{mol}$)(Zhang et al., 1990) using the formula

> $P_{f} = [V_{0} \times d(V/V_{0})/dt]/[S \times V_{w} \times (\operatorname{osm}_{in} - \operatorname{osm}_{out})]$ (Eq. 1)

Obcyte Membrane Isolation and Immunoblot Analysis-Total oocyte

mutagenesis and domain exchanges in this study. SPLICE 1 is the splice site for MIP-CHIP-1 and CHIP-MIP-1; SPLICE 2 is the splice site for MIP-CHIP-2 and CHIP-MIP-2. The proline introduced in CHIP at position Asp-131 is indicated with #. NPA motifs are indicated with asterisks. Filled symbols indicate amino acids that are conserved in more than half of the members of the MIP family from bacteria, yeast, plants and animals (Reizer et al., 1993).

carboxyl-terminal amino acids of bovine MIP (PEVTGEPVELKTQAL) (Gorin et al., 1984) was coupled to keyhole limpet hemocyanin. Rabbits were injected with 400 μ g of conjugated synthetic peptide mixed with Freund's complete adjuvant. After 4 weeks, and every 3 weeks thereafter, the rabbits were boosted with 200 μ g of conjugated synthetic peptide mixed with incomplete Freund's adjuvant. The produced anti-MIP antiserum was tested for specificity and cross-reactivity by an enzyme-linked immunosorbent assay.

Immunolocalization of MIP in Xenopus Oocytes—Oocytes were frozen for immunofluorescence microscopy in Tissue-Tek mounting medium (Miles Inc.). Sections of 5 μ m were cut by cryostat, collected on gelatin-coated slides, and fixed at room temperature for 5 min in 1% (w/v) periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974). Sections were washed 3 times with TBS (0.9% NaCl, 25 mm Tris, pH 7.4) and incubated overnight at 4 °C with anti-MIP antibody at a dilution of 1:1,000 in TBS containing 10% (v/v) goat serum. After 3 washes with TBS, the sections were incubated for 1 h at 37 °C with affinity-purified fluorescein isothiocyanate-labeled goat-anti-rabbit IgG (Sigma Immuno Chemicals) at a dilution 1:50 in TBS containing 10% (v/v) goat serum. After 3 more washes in TBS, the sections were embedded in Mounting Medium (Sigma) and analyzed by immunofluorescence microscopy. Electrophysiology-Studies were carried out as described (Preston et al., 1992) using a two-microelectrode voltage clamp with Clampex software. Water- and cRNA-injected oocytes were voltage-clamped at a holding membrane potential of -70 mV, repolarized to -90 mV, and

membranes (Preston et al., 1993) and plasma membranes (Wall and Patel, 1989) were isolated from groups of 4-30 occytes, solubilized in 1,25% (w/v) SDS at 60 °C for 10 min, electrophoresed into 12% SDSpolyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose (Davis and Bennett, 1984), incubated with a 1:10,000 dilution of anti-MIP antibody, or a 1:1000 dilution of anti-CHIP antibody (Smith and Agre, 1991), and visualized using the ECL Western blotting detection system (Amersham Corp.). Molecular weights were determined relative to the mobility of prestained SDS-PAGE standards (Bio-Rad). Anti-MIP Antibody-A synthetic peptide corresponding to the 15

W-glycosylation had still not occurred (not shown). Oocytes expressing MIP-CHIP-1 and CHIP-MIP-1 chimeras did not exhibit a significant increase in P_f above water-injected controls, suggesting that these chimeric proteins cannot form functional water channels. Oocytes expressing MIP-CHIP-2 also exhibited P_f s similar to control oocytes. In contrast, oocytes expressing CHIP-MIP-2 were found to have P_f s similar to oocytes expressing wild-type CHIP (Fig. 6), indicating that the fere with the water-transporting domain of MIP does not interfere with the water-transporting domains of CHIP.

Single Amino Acid Substitutions—Unlike CHIP, the inblocked by HgCl₂. A putative mercury-sensitive cysteine residue was introduced into MIP at position 181, which conforms to the mercury-sensitive cysteine 189 in CHIP, but the substitu-

Water Permeability of MIP—Osmotic water permeability (P_{i}) ·(UMOUS process for water-injected oocytes was too slow for analysis (not decreased again to the initial values, although the shrinking buffer to cause shrinkage to the initial volume, the conductance increase in conductance. After replacing ocytes in isotonic with water, MIP cRNA, or CHIP cRNA exhibited a similar membrane currents were again measured. Oocytes injected medium, and, after being osmotically swollen to a similar size, CHIP CRNA (Fig. 3). The occytes were placed in hypotonic were measured in oocytes injected with water, MIP cRNA, or the absence of osmotic swelling, no differences in conductance expressing MIP with a two-microelectrode voltage clamp. In was tested by measuring the conductance of oocyte membranes lennsdo noi ns ai AIM tadt aizedtoqyd edT. (0661, .1b te gairdA) planar lipid bilayers forms channels with high conductance Conductonce-It was reported that MIP reconstituted into Reted to the plasma membrane (Eig. 2B). a single strong signal at 27 kDa, confirming that MIP is tarblot analysis of isolated ocyte plasma membranes also showed plasm showed only weak immunostaining (Fig. 2A). Immunoimmunolabeling of the plasma membrane, whereas the cyto-Oocytes injected with MIP dRMA showed a strong anti-MIP studied by immunofluorescence microscopy of frozen sections. tion of the protein into the oocyte plasma membrane was -rearin and Procedures"). Expression of MIP and insert ("serubesory IstnemineqxE" ees) measurement of their functional activity by a swelling assay the standard system for expression of water channels and Immunolocalization—Oocytes from X. laeus have provided

(UMOUS with oocytes expressing native MIP and CHIP proteins (not were identified, and the levels of expression were comparable analyzed by immunoblot, polypeptides of the anticipated size pranes of oocytes expressing each of these constructs were terminal cytoplasmic domains of the molecules. When mem-CHIP-MIP-2 chimeras are spliced at a site in the carboxyltandem repeats are normally joined. The MIP-CHIP-2 and terminal half of the other spliced at a site in loop C where the the amino-terminal half of one molecule and the carboxylof CHIP. The MIP-CHIP-1 and CHIP-MIP-1 chimeras contain A qool and mori 16-46 seubiser and yd beasiger A gool ni 64-66 (loop A) chimera represents a MIP molecule with residues consequences were measured (Table I, Fig. 1). The MIP-CHIP teristics of the two proteins were exchanged, and the functional that might be of importance for the specific functional charac-AIHO DUB AIM JO SUIGMOU-SUISTONA AIHO AIM SIJSMIND (IOm/Isox 81 bms 01 =

Unlike all of the known Aquaporins, MIP does not contain a potential *N*-glycosylation site in either loop A or loop C, and no glycosylation of MIP was detected when the polypeptide was expressed in oocytes (Fig. 2B). The MIP-CHIP (loop A) construct contains the CHIP glycosylation site at residue asparagine 42 in loop A. Despite the presence of a potential glycosyla-tion site, oocytes expressing this construct still did not exhibit tion site, oocytes expressing this construct still did not exhibit tion site, oocytes in P_f , and immunoblots revealed a 27-kDa polypeptide tide but not higher molecular mass bands, indicating that

Fic. 2. A, immunofluorescence microscopy of control oocytes and oocytes expressing MIP. Frozen sections were prepared from *Xenopus* of MIP cRNA and probed with anti-MIP antibody (see "Experimental Procedures"). Note strong labeling of plasma membrane and negligible total protein (TP, equivalent of 0.2 oocytes) and plasma membranes total protein (TP, equivalent of 0.2 oocytes) and plasma membranes polyacrylamide gel electrophoresis immunoblot was incubated with a polyacrylamide gel electrophoresis immunoblot was incubated with a polyacrylamide gel electrophoresis immunoblot was incubated with a

depolarized to +50 mV in 20 mV atep intervals. The membrane potential was returned to the holding potential between each atep.

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pendent experiments. Water transport through aqueous channels is characterized by a low Arrhenius activation energy, which is equivalent to diffusion of water in bulk solution $(E_A \sim 4 \text{ kcal/mol})$, whereas a high value $(E_A > 10 \text{ kcal/mol})$ (Finkelstein, 1987). Water permeability of oocytes injected with 25 ng of MIP cRNA was mater transport, the activation energy was low $(E_A = 3.9 \text{ kcal/}$ mol $\pm 1.4 \text{ kcal/mol}$, average of 4 experiments, Fig. 5). Indicative of diffusional water movement, the activation energy was low $(E_A = 3.9 \text{ kcal/}$ mol $\pm 1.4 \text{ kcal/mol}$, average of 4 experiments, Fig. 5). Indicative of diffusional water movement, the activation energies of mol $\pm 1.4 \text{ kcal/mol}$, average of 4 experiments ($E_A = 3.9 \text{ kcal/}$ mol $\pm 1.4 \text{ kcal/mol}$, average of 4 experiments, Fig. 5). Indicative mol $\pm 1.4 \text{ kcal/mol}$, average of 4 experiments ($E_A = 3.0 \text{ kcal/}$ mol $\pm 1.4 \text{ kcal/mol}$, average of 4 experiments ($E_A = 0.0 \text{ kcal/}$).



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of Xenopus oocytes was measured in hypotonic medium 3 days after injection with 50 nl of water or 10 ng of MIP or CHIP CRNA. Water-injected control oocytes swelled minimally ($P_f = 10 \pm 3.2 \ \mu m/s$). Oocytes injected with MIP cRNA displayed a control oocytes ($P_f = 45 \pm 8.2 \ \mu m/s$). A more than 30-fold increase in P_f was exhibited by oocytes injected with the same amount of CHIP cRNA ($P_f = 351 \pm 43 \ \mu m/s$, Fig. 4, A and B). Although the P_f of MIP-injected oocytes was only ~15% of CHIP-injected oocytes the MIP-injected oocytes had significantly greater P_f than the control oocytes (p < 0.05, Fig. 4A), a difference that was consistently reproduced in each of 14 indedifference that was consistently reproduced in each of 14 inde-





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FIG. 3. Mombrane ion conductance. Voltage clamp was used to measure currents at voltages from -90 mV to +50 mV of water-injected control oocytes, oocytes injected with 25 ng of MIP cRNA, or oocytes injected with 0.5 ng of CHIP cRNA. The currents were determined in 200 mosm modified Barth's solution. The oocytes were swollen by replacing modified Barth's solution with a hypotonic medium, and current measurements were repeated. Control oocytes were placed in H₂O, and the current was measured after 30 min. Oocytes expressing MIP and CHIP were placed in modified Barth's solution diluted from 200 to 70 mosm, and currents were measured after 20 and 30 s. Oocytes were shrunk back to their normal volume by replacing the hypotonic medium with isotonic modified Barth's solution, and currents in oocytes expressing MIP and CHIP were measured after 8 min.

tion MIP(A181C) did not confer mercury sensitivity to MIP (Fig. 7). The cysteine at position 14 in the amino-terminal domain of MIP was replaced by a valine, since other Aquaporins do not contain a cysteine at this position, but the P_f of (14V mutant MIP was not different from wild-type MIP (Fig. 8A). MIP contains a proline in the second extracellular loop (residue 123 in loop C), a site where other members of the MIP family do not contain this residue. Introducing a proline at this position in CHIP (D131P) also did not change the P_f (Fig. 8A).

All known Aquaporins except AQP4 contain a proline in the 5th transmembrane segment; no proline exists in the 5th transmembrane domain of MIP. When a proline was introduced in MIP (V160P), the substitution reproducibly enhanced the P_f by $50 \pm 20\%$ relative to the P_f of occytes expressing wild-type MIP (Fig. 8A). Notably, the amount of MIP protein expressed in occytes was not different after injection of equal amounts of MIP cRNA and MIP(V160P) cRNA (Fig. 8B). Although not large, this increase in P_f was confirmed in each of five different experiments. A "gain of function" mutation suggests that the P_f of MIP can be increased by introducing subtle conformational changes in the molecule. Nevertheless, replacing the proline in CHIP at this position (P169A) to the corresponding residue of MIP had no measurable influence on the P_f of CHIP (Fig. 8A). These results show that MIP exhibits a low P_f value that can be increased by introducing subtle changes in the molecule.

One explanation may be that MIP needs to undergo an activation or structural rearrangement to function as a water channel, whereas CHIP is constitutively in the activated state. Two sites in the carboxyl-terminal cytoplasmic domain of MIP are phosphorylated in vivo (Lampe and Johnson, 1990). Since these sites are not conserved in other members of the MIP family, the sites may play a role in regulation of MIP function. Mutation of the two putative phosphorylation sites to the corresponding residues in CHIP (S243V, S245E) slightly decreased the P_{f} (Fig. 8A), but the amount of protein expressed was comparably reduced as compared with wild-type MIP by immunoblot (Fig. 8B). The possibility of stretch-activation was also considered, but if it exists, it was not reproduced by simple increase in volume, since the rate of osmotic swelling did not increase with time (Fig. 9). Thus, if an activation step confers CHIP-level water permeability on MIP, the identity of this step remains

unknown.

DISCUSSION

Even though the cDNA encoding MIP was cloned more than a decade ago (Gorin *et al.*, 1984), the physiological roles of the protein are still not understood. Detailed studies of the homologous proteins MIP (Ehring *et al.*, 1990), Nodulin-26 (Weaver *et* 9014

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50 100 150 200 250300 350 400 450 Q $P_r (\mu m/s)$

 E_A was determined by measuring the P_f at 10, 20, and 30 °C. Control oocytes were injected with water and MIP oocytes were injected with 25 ng of MIP cRNA. In this experiment, each point represents 7-10 control oocytes or 11-15 MIP oocytes.

FIG. 4. A, increased osmotic water permeability of Xenopus oocytes expressing MIP and CHIP. Oocytes were injected with water or 10 ng of in vitro transcribed cRNA encoding MIP or CHIP. After 72 h, P_{f} s were determined by video microscopic measurement of the rate of swelling after transfer to hypotonic medium. Shown are the means \pm S.D. for 8 oocytes. B, time-dependent osmotic swelling of oocytes injected with water or 10 ng of cRNA encoding MIP or CHIP. Shown are the means from five traces \pm S.E.

al., 1994), and preliminary studies with CHIP^2 revealed large voltage-dependent conductances when reconstituted into planar lipid bilayers. Nevertheless, the magnitude of the currents in bilayers containing MIP was far above the conductances measured in normal lens (Mathias et al., 1979, 1991). Therefore, while the planar lipid bilayer studies are highly reproducible, their relevance to normal lens physiology remains uncertain. Moreover, the studies reported here (Fig. 3) and preliminary studies of other investigators (Kushmerick et al., 1994) failed to confirm ion conductance by MIP expressed in oocytes.

FIG. 6. Osmotic water permeability of oocytes expressing chimeric MIP-CHIP proteins. P_f was determined from oocytes injected with water or 10 ng of cRNA encoding MIP, CHIP, or the indicated MIP-CHIP chimeric proteins. Shown are the means \pm S.D. of five ocytes. *, p < 0.05 compared with water-injected control ocytes.

meability of AQP3 (Ma et al., 1994), a protein found by two other groups to exhibit high P_f comparable with the other Aquaporins (Ishibashi et al., 1994; Echevarria et al., 1994). The studies reported here document that oocytes expressing MIP exhibit osmotic water permeabilities 4-5-fold above control oocytes (Fig. 4) with activation energies identical to the Aquaporins (Fig. 5). These observations are supported by preliminary studies of other investigators who also found an increase in P_f of oocytes expressing frog MIP (Kushmerick *et al.*, 1994) or bovine MIP (Chandy et al., 1995). MIP may contribute to the maintenance of lens transparency by enhancing uptake of intercellular water by adjacent lens fiber cells. The narrow geographic separation of lens fiber cells (which contain MIP) and lens epithelial cells (which contain CHIP) suggests functional cooperativity. Osmotic gradients provide the driving force for Aquaporin-mediated water transport in kidney and most other tissues (Nielsen et al., 1993a, 1993b). It is likely that hydrostatic forces move water through CHIP in endothelium of the proximal capillary bed (Nielsen et al., 1993b), and a related process may occur through MIP when the lens shape is rapidly altered by contraction of muscles in the ciliary body to provide fine focus of the corneal image upon the retina. A molecular explanation for why MIP is a weaker water

Soon after the discovery that CHIP is a molecular water channel (Preston et al., 1992), several labs evaluated MIP and other homologous proteins for osmotic water permeability. One group reported that MIP is not a water channel (Verbayatz et al., 1994), but apparently these investigators used the same techniques with which they failed to detect osmotic water per-

² J. Hall and P. Agre, unpublished observations.

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occytes expressing CHIP, MIP, or MIP (A181C). Occytes were injected with 1 ng of CHIP cRNA or 10 ng of MIP cRNA or 10 ng of MIP(A181C) cRNA. P, was determined without pretreatment (open bars) or after 5 min in buffer containing 3 mM HgCl₂, followed by swelling in the presence of HgCl_2 (solid bars). Shown are the means \pm S.D. of five oocytes.

Fig. 9. Time-dependent osmotic swelling of oocytes expressing MIP. 3 days after injection of 25 ng of MIP cRNA, oocytes were transferred to 20 mosM modified Barth's solution, and swelling was recorded until the oocyte burst (X). Shown are the means from 7 traces \pm S.E.

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Fig. 10. Evolutionary tree of MIP and the mammalian Aqua-

MIP was expressed well in occytes and was targeted to the plasma membrane (Fig. 2). Neither the phosphorylation sites nor the other residues in the carboxyl-terminal cytoplasmic domain of MIP appeared to function as restraints (Figs. 6 and 8), and several different residues and domains from CHIP were spliced into MIP without increasing the P_f (Figs. 6-8). Despite introduction of an N-glycosylation site, MIP-CHIP (loop A) was still not glycosylated, and introduction of the potential mercurial inhibition site in MIP (A181C) failed to confer sensitivity (Fig. 7). Likewise, when a cysteine was introduced into the mercury-insensitive AQP4, the recombinant protein also failed to demonstrate mercury-inhibitable P_f (Jung et al., 1994b), implying that MIP and AQP4 are structurally different from CHIP near the extracellular side of the aqueous pore. Nevertheless, substitution of proline in the 5th bilayer-spanning domain yielded a 50% increase in P_f (Fig. 8). This intriguing gain of function suggests that simple alterations in the bilayerspanning domains can alter the conformation of MIP to more closely resemble CHIP. No loss of function was detected in the corresponding substitution (P169A) in CHIP; however, a small decrease in a high P_f should be more difficult to measure than a comparable increase in a low value. An alternative explanation may be that MIP requires a specific membrane organization, such as formation of orthogonal arrays, or a particular membrane environment that may be poorly reproduced in the oocyte expression system. For example, oocytes do not have

Fig. 8. Osmotic water permeability of occytes expressing MIP and CHIP mutants. A, occytes were injected with water or 25 ng of eRNA encoding MIP, MIP(C14V), MIP(V160P), MIP(S243V, S245E), or 5 ng of eRNA encoding CHIP, CHIP(D131P), or CHIP(P169A). The P_f was measured 3 days after injection. Shown are the means \pm S.D. *, \dot{p} < 0.05 compared to water-injected occytes, **, p < 0.05 compared with wild-type MIP. B, immunoblot comparison of total protein (TP, equivalent of 0.2 occytes) or plasma membranes (PM, equivalent of 20 oocytes) from oocytes expressing MIP or the indicated MIP mutants. SDS PACE immunoblot was incubated with a polyclonal antibody speeffe for the carboxyl terminus of MIP.

transporter than the other Aquaporins was sought since the amino acid sequences of MIP and the Aquaporins are strikingly similar (Preston and Agre, 1991; Reizer et al., 1993)) (Fig. 10).

plasma membranes with wavy junctions similar to lens fiber cells (Zampighi *et al.*, 1989), and if this is important to the function of MIP, the water permeability studies may yield spuriously low values in oocytes.

Similar to other bilayer-spanning proteins, MIP may have multiple physiological functions, and it is possible that the primary role of MIP is not water transport. The red cell band 3 protein is the membrane anion exchanger (AE1), a cytosolic regulator of glycolytic enzyme activity, and the structurally important attachment site for ankyrin on the membrane (for review, see Low (1986)). MIP may also have a structural function, since the protein has been shown to enhance adhesion with membranes containing negatively charged phospholipids (Michea et al., 1994). Also, it is known that some proteins are expressed in lens where their function is unrelated to their functions in other tissues (e.g. crystallins, for review, see Piatigorsky and Wistow (1989) and De Jong et al. (1994)). Thus the extremely high expression of MIP in lens may be far above the level needed for water permeability, since the abundance may be needed for an unrelated function. A mutation has been identified in mice that may provide insight into other potential functions of MIP, since these mice develop cataracts prior to birth (Muggleton-Harris et al., 1987). The *cat* mouse mutation results in lower abundance of MIP mRNA with the major transcript being truncated, and MIP was not detectable in lens by immunocytochemistry (Shiels and Griffin, 1993). The mutation is expressed as a dominant trait and has been mapped to the distal end of chromosome 10 (Muggleton-Harris et al., 1987), coincident with the MIP locus (Griffin and Shiels, 1992). Mutations in genes encoding structural proteins usually produce dominantly inherited disorders, whereas mutations in transporters such as the CFTR are recessively inherited. Consistent with this, mutations in the AQP2 gene were recently identified in homozygotes and a compound heterozygote with severe nephrogenic diabetes insipidus, while the heterozygous relatives were unaffected (Deen et al., 1994; van Lieburg et al., 1994). Careful histological analysis of the early stages of disease in the cat mouse may provide clues to the critical function of MIP, which is the first defect leading to the development of cataracts in this animal model.

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