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Water Channel Properties of Major Intrinsic Protein of Lens*

(Received for publication, December 27, 1994)

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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 Xenopus 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\textsubscript{o}) of MIP oocytes was increased 4–5-fold with a low Arrhenius activation energy, while the P\textsubscript{o} of CHIP oocytes increased >30-fold. To identify structures responsible for these differences in P\textsubscript{o} 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\textsubscript{o} 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)\textsuperscript{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 Ca\textsuperscript{2+} and calmodulin (Nikaido and Rosenberg, 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 gap-junction 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 water-selective membrane channels and are now referred to as the "Aquaporins" (Chrişteş and Agre, 1994). The purification and cDNA cloning of the 28-kDa channel-forming integral membrane protein, CHIP (Denker et al., 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; Echavarria 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.

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 Mutagen phagemid in vitro mutagenesis kit (Bio-Rad). Table I lists the
Table 1

<table>
<thead>
<tr>
<th>MIP constructs</th>
<th>Wild-type amino acid/codon</th>
<th>Mutant amino acid/codon</th>
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</thead>
<tbody>
<tr>
<td>MIP (V112Bam)</td>
<td>Val-112/GTC</td>
<td>Val-Asp-Pro/GTggatccC</td>
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<tr>
<td>MIP(C14V)</td>
<td>Cys-14/TGT</td>
<td>Val-14/GYT</td>
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<tr>
<td>MIP(V160P)</td>
<td>Val-160/GTG</td>
<td>Pro-160/CCA</td>
</tr>
<tr>
<td>MIP(A181C)</td>
<td>Ala-181/GCA</td>
<td>Cys-181/TGT</td>
</tr>
<tr>
<td>MIP(S245V/S246E)</td>
<td>Ser-Glu-Ser-245/AGTgatTCC</td>
<td>Val-Glu-Glu/GTTgagGAA</td>
</tr>
<tr>
<td>CHIP constructs</td>
<td>Wild-type amino acid/codon</td>
<td>Mutant amino acid/codon</td>
</tr>
<tr>
<td>CHIP(T120Bam)</td>
<td>Thr-120/ACT</td>
<td>Thr-Asp-Pro/AACggatccT</td>
</tr>
<tr>
<td>CHIP(D131P)</td>
<td>Aap-131/GAT</td>
<td>Pro-131/CCT</td>
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<tr>
<td>CHIP(P169A)</td>
<td>Pro-169/CCC</td>
<td>Ala-169/GCC</td>
</tr>
<tr>
<td>MIP-CHIP (loop A)</td>
<td>MIP(1-32)-CHIP(34-51)-MIP(44-263)</td>
<td>270</td>
</tr>
<tr>
<td>MIP-CHIP-1</td>
<td>MIP(1-112)-CHIP(121-269)</td>
<td>263</td>
</tr>
<tr>
<td>MIP-CHIP-2</td>
<td>MIP(1-226)-CHIP(235-269)</td>
<td>261</td>
</tr>
<tr>
<td>CHIP-MIP-1</td>
<td>CHIP(1-120)-MIP(133-263)</td>
<td>273</td>
</tr>
<tr>
<td>CHIP-MIP-2</td>
<td>CHIP(1-234)-MIP(227-263)</td>
<td>271</td>
</tr>
</tbody>
</table>

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 CHIP-MIP-2 and CHIP-MIP-2 were constructed using the megaprimer polymerase chain reaction method (Bunk and Galinski, 1991) exchanging at Arg-226 of MIP and Arg-234 of CHIP, using the following antisense primers: MIP-CHIP-2, 5′-CTCAGAAACACTCTTGAGC-CGTGGGGC-CA4(A)TGAGG-3′; CHIP-MIP-2, 5′-CTCAAGAACACTCTTGAGC-GTGGGGG-GCA4(A)TGAGG-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-substitution oligonucleotide primer (not shown) in a site-directed mutagenesis reaction. All mutations were confirmed by enzymatic nucleotide sequencing (U. S. Biochemical Corp.).

Capped RNA transcripts were synthesized in vitro using T3 RNA polymerase with Xhol-digested MIP, CHIP, or mutant expression vector DNA, and the RNA was purified as described (Yisraeli and Melton, 1989). Preparation of Oocytes and Measurement of Pf—Female Xenopus laevis were anesthetized on ice, and stage V and VI oocytes were removed and prepared (Lu et al., 1990). The day after isolation, oocytes were injected with either 50 nl of water or 0.5–25 ng of cRNA in 50 nl of water. Injected oocytes were maintained for 2–3 days at 18 °C prior to osmotic swelling, membrane isolation, or voltage clamp experiments. Oocyte swelling was performed at 22 °C following transfer from 200 mosM (osmout), to 70 mosM (osmout) CHIP or either 70 or 20 mosM (osmout) MIP modified Barth’s solution diluted with water. Sequential oocyte images were digitized at 8-s intervals for a total of 1 min, and the volumes of the sequential images were calculated as described (Preston et al., 1983). The change in relative volume with time, dV/V0 dt, was fitted by computer to a quadratic polynomial, and the initial rates of swelling were calculated. The osmotic water permeability (Pw, mm/s) was calculated from osmotic swelling data between 5 and 10 s, initial oocyte volume (V0 = 8 x 10^-4 cm^3), initial surface area (S = 0.045 cm^2), and the molar ratio of water (Vwater = 18 cm^3/mol)(Zhang et al., 1990) using the formula:

\[ P_w = \frac{V_o \times d(V/V_0)/dt}{(S \times V_o - \text{osm}_\text{in} - \text{osm}_\text{out})} \] (Eq. 1)

Oocyte Membrane Isolation and Immunoblot Analysis—Total oocyte membranes (Preston et al., 1993) and plasma membranes (Wall and Patel, 1989) were isolated from groups of 4–30 oocytes, solubilized in 1.25% (w/v) SDS at 60 °C for 10 min, electrophoresed into 12% SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose (Towbin and Burnesi, 1984), incubated with a 1:10,000 dilution of anti-MIP antibody, or a 1:1,000 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 carboxyl-terminal amino acids of bovine MIP (PETYGFVPVELKQTAL) (Gorin et al., 1984) was coupled to keyhole limpet hemocyanin. Rabbits were injected with 400 µg of conjugated synthetic peptide mixed with Freund’s complete adjuvant. After 4 weeks, and every 3 weeks thereafter, the rabbits were boosted with 200 µg 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 µm were cut by cryostat, collected on gelatin-coated slides, and fixed at room temperature for 6 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% (w/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% (w/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., 1993) 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...
When permeability of MIP—osmotic water permeability (p)

shows no water-regulated opening was too slow for flow (Q) to
become apparent in the initial stage, although the
separation between the initial opening and
the sudden increase in water flow is not
visible in CHN of MIP CRNA.

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Water Channel Properties of MIP

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Fig. 3. Membrane 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.

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 Pf (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
Water Channel Properties of MIP

**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* values were determined by video microscopic measurement of the rate of swelling after transfer to hypotonic medium. Shown are the means ± 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 ± S.E.

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 (Verbavatz et al., 1994), but apparently these investigators used the same techniques with which they failed to detect osmotic water permeability of AQP3 (Ma et al., 1994), a protein found by two other groups to exhibit high *P* 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* 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 channel is that it is a homodimer. CHIP from different species exhibits 43% sequence identity with MIP, and 55% identity with MIP-CHIP proteins. These observations are supported by preliminary studies of other investigators who also found an increase in *P* 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.

**Fig. 5.** Arrhenius activation energy (*Eₐ*) of osmotic water permeabilities of control oocytes and oocytes expressing MIP. The *Eₐ* was determined by measuring the *P* 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. 6.** Osmotic water permeability of oocytes expressing chimeric MIP-CHIP proteins. *P* 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 ± S.D. of five oocytes. *, *p < 0.05 compared with water-injected control oocytes.

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*J. Hall and P. Agre, unpublished observations.*
MIP was expressed well in oocytes 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., 1994a), 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 bilayers-spanning domain yielded a 50% increase in $P_f$ (Fig. 8). This intriguing gain of function suggests that simple alterations in the bilayers-spanning 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
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 lenses 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 lenses 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 lenses 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.

Acknowledgments—We gratefully thank A. Hartog for performing immuno-cytochemistry of MIP in oocytes and Dr. R. J. M. Bindels for help in formulating a computer routine to analyze swelling curves of oocytes. We also thank James Hall, Richard Mathias, Paul Lampe, and David Beebe for valuable discussions.