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CHAPTER 1

Discovery of the Aquaporins and Their Impact on Basic and Clinical Physiology

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I. PRE-AQUAPORIN ERA

Water channels have become increasingly well recognized during the past 10 years; however, the basic issues of membrane water permeability were recognized much earlier.

A. Water Permeation of Cell Membranes

The combined efforts of multiple laboratories indicated that water permeation is a feature of some but not all cells and tissues. Since the time of August Krogh and his studies of capillary permeability, physiologists at the University of Copenhagen have addressed the problem of how water and solutes are transported across epithelia. Hans Ussing and colleagues studied amphibian skin and recognized that certain epithelia are exceedingly permeable, hence "leaky" (reviewed by Ussing, 1965). Other investigators followed these studies with electron microscopic analyses, which indicated that water channels ("aggrephores") must reside within tissues such as amphibian bladder (reviewed by Kachadorian *et al.*, 2000), and when stimulated appropriately, the vesicles were believed to traffic to the cell surface, inducing water permeability. This process was reversed by reinternalization of the putative water channels, and the "shuttle hypothesis" became widely recognized (Wade *et al.*, 1981).

A. K. Solomon and colleagues concentrated their attention on red blood cells. They measured large and selective water fluxes through the membranes with low Arrhenius activation energies, indicating that porelike molecules must reside in the plasma membranes (reviewed by Solomon, 1968). Taking this further, Robert Macey and colleagues observed that membrane water permeability is selectively inhibited by $HgCl_2$ and certain organomercurials and that water permeability could be restored by treating the red cells with reducing agents (reviewed by Macey, 1984). Thus, the water pores must be formed from proteins with free sulfhydryls that react with mercurials.

Fortunately, these observations were integrated by Alan Finkelstein in a monograph titled *Water Movements through Lipid Bilayers, Pores, and Plasma Membranes—Theory and Reality* (1987). In this comprehensive treatise, Finkelstein posed the question "Who are the water pores?" To answer this, he considered the possibility that one of the known red cell proteins must be the long-sought water channel, because his biochemist friends (stated to be "few in number") assured him that all the major membrane proteins must certainly have already been discovered by then. In retrospect, Finkelstein pointed out that the identity of the membrane water pores was certainly not known. Attempts to identify molecular water channels were made by several laboratories using various approaches: labeling of red cell anion transporter with isotopic mercury (Solomon *et al.*, 1983); measuring

increased osmotic water permeability of oocytes expressing glucose transporter protein (Fischbarg *et al.*, 1990); functional evaluation of candidate proteins from amphibian bladder (Harris *et al.*, 1992); and attempts to clone by expression (Zhang *et al.*, 1990). Unfortunately, none of these direct approaches was successful.

B. Red Cell 28-kDa Membrane Protein

It has been said that "hypothesis-driven research is highly overrated" (A. G. Gilman, personal communication, 2000), suggesting that unexpected results may sometimes yield insights that are much greater than anticipated and may change a field altogether. The surprising discovery of the 28-kDa red cell membrane protein, now known as AQP1, is a clear demonstration of the importance that a chance observation can make in a field stymied by logical approaches.

The Rh blood group antigens are of large clinical importance. Their molecular identities, however, have long resisted identification. Utilizing radiochemically labeled red cell membranes from Rh(D) positive red cells, a 32-kDa membrane protein was isolated by hydroxylapatite chromatography (Agre *et al.*, 1987; Saboori *et al.*, 1988). This protein is virtually invisible to staining with Coomassie blue; however, in pure form the protein is well visualized with silver reagent. A 28-kDa polypeptide was also unstained by Coomassie but was visualized with silver reagent (explaining why Professor Finkelstein's biochemist friends failed to see it). The 28-kDa polypeptide was initially believed to be a proteolytic fragment of the 32-kDa Rh polypeptide. Surprisingly, antibodies raised in rabbits only reacted with the 28-kDa band, which was subsequently characterized and found to be unrelated to Rh (Denker *et al.*, 1988). Although initially believed to be linked with the membrane skeleton, the 28-kDa polypeptide could be solubilized in higher concentrations of nondenaturing detergent. The relative insolubility of the 28-kDa polypeptide in the detergent *N*-lauroylsarcosine, a substance once well known to the baby boomer generation as Gardol, the secret ingredient of Colgate's toothpaste, proved to be extremely helpful. Virtually all other red cell membrane proteins can be removed in one step, leaving nearly pure 28-kDa polypeptide. This procedure permitted isolation of pure 28-kDa polypeptide in milligram concentrations (Fig. 1).

Several features of the 28-kDa polypeptide suggested that it may have a channel-like structure and resulted in the temporary designation "CHIP28," for channel-like integral protein of 28 kDa (Smith and Agre, 1991). When analyzed in nonionic detergent, the protein exhibited filtration and velocity sedimentation properties indicating that it is a multisubunit oligomer, predicted to be a homotetramer. Most of the protein resides between the leaflets of the lipid bilayer, and a short N terminus and the C terminus were localized to the cytoplasmic space. Unlike other membrane proteins, a complex polylactosaminoglycan was attached to only one of the

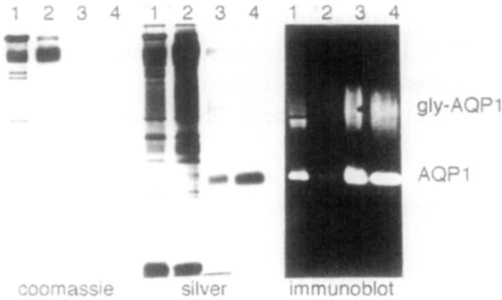


FIGURE 1 Purification of AQP1 protein from human red blood cells. (*Left*) SDS–PAGE stained with Coomassie; (*middle*) same stained with silver; (*right*) immunoblot reacted with anti-AQP1. Lane 1, whole red cell membranes; lane 2, *N*-lauroylsarcosine-soluble proteins; lane 3, *N*-lauroylsarcosine-insoluble proteins; lane 4, FPLC purified AQP1. Note 28-kDa unglycosylated and 40- to 60-kDa glycosylated protein. Reproduced with permission from *Methods in Enzymology* (Agre *et al.*, 1998b).

four subunits. The amino acid sequence of the first 35 N-terminal residues was determined by Edman degradation protein sequencing. Interestingly, the sequence was observed to be related to major intrinsic protein of lens (MIP), an abundant but functionally undefined protein (Gorin *et al.*, 1984). The 28-kDa protein was noted to be extremely abundant in red blood cells (approximately 200,000 monomers per membrane), in apical brush border of renal proximal tubules (approximately 4% of the total membrane protein), and in descending thin limbs of Henle's loop (Denker *et al.*, 1998). Recognizing that these proteins are all highly permeable to water, the late John C. Parker (1936–1993), a noted membrane physiologist at the University of North Carolina at Chapel Hill, first suggested that the 28-kDa polypeptide may be the long-sought water channel (Parker and Agre, personal discussions, 1990). As will be documented in this volume, Parker's suggestion was to have major consequences for the direction of water transport research.

II. THE FIRST RECOGNIZED WATER CHANNEL PROTEIN

As with other physiologically important molecules, identification of the first member of the water channel family led to increased interest in this area of biology.

A. cDNA Cloning

The N-terminal sequence of CHIP28 was utilized to design oligonucleotides that were used clone the cDNA from an erythroid library (Preston and Agre, 1991). This was done in stages, and confirmation that the product achieved was obtained by demonstrating that the expressed protein reacted with the specific antibody

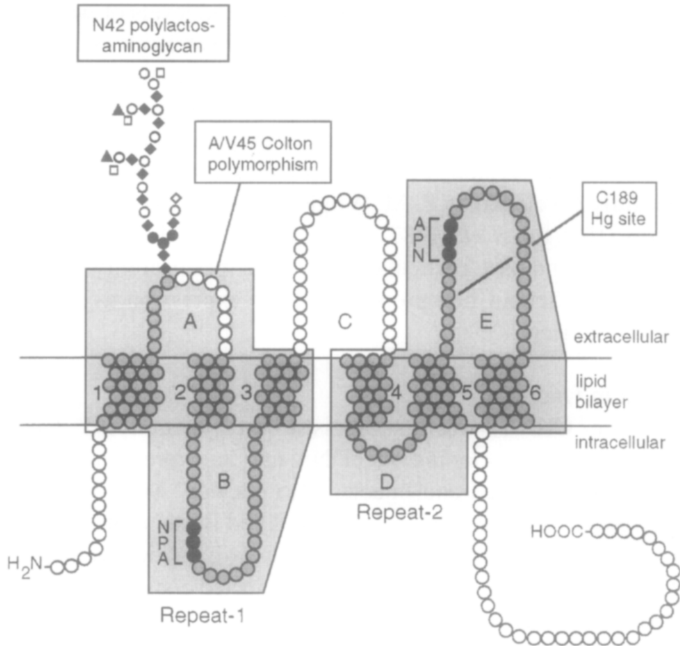


FIGURE 2 Schematic diagram of AQP1 monomer showing sequence repeats and selected structural features. Reproduced with permission from *Journal of Structural Biology* (Heymann *et al.*, 1998).

reactive with the C terminus. Sequence analysis demonstrated that the protein contains a tandem repeat of two related peptides, each encoding three presumed bilayer-spanning domains (Fig. 2). A potential glycosylation site was noted in the extracellular loop A, and two highly conserved loops were noted to contain the preserved motif Asn-Pro-Ala (NPA).

Other clues to possible function of this protein were observed at this time. The GenBank contained related sequences encoding a microbial homolog and sequences encoding four plant homologs. Of particular interest, expression of a homolog from pea plants (TUR) was noted to become induced by water deprivation (Guererro *et al.*, 1990). Moreover, the size of the 28-kDa polypeptide was close to the size of the membrane water channel independently determined by radiation inactivation studies (van Hoek *et al.*, 1991).

B. Functional Studies

Unlike measurement of ion currents or transport of radioisotopically labeled solutes, the measurement of membrane water transport poses special difficulties (Solomon, 1989; Verkman, 1992). Because all membranes exhibit some degree of

diffusional water permeability, significant background levels of water permeability are always present. Likewise, the movement of water across cell membranes is very fast and reaches equilibrium in milliseconds. Thus, the use of isotopically labeled water (^3H_2O) is not generally helpful because hydrogen ions are freely exchangeable with other water molecules and isotopic studies are not amenable to fast measurements. Expression of transport proteins in *Xenopus laevis* oocytes had been successfully developed by E. M. Wright and colleagues to clone other transport processes (Hediger *et al.*, 1987). Recognizing that these cells are relatively easy to manipulate and have particularly low water permeability, Eric Windhager at Cornell Medical School first proposed oocyte expression as a mechanism to study water transporters.

1. Expression in Oocytes

The function of CHIP28 was first assessed by expression in *X. laevis* oocytes (Preston *et al.*, 1992). By cloning the cDNA into an expression vector, complementary RNA was prepared and injected into oocytes. After 3 days in culture, the oocytes were transferred from isotonic modified Barth's solution (200 mos*M*) to diluted solution (70 mos*M*). This resulted in a dramatic swelling and explosion in the test oocytes, whereas control water-injected oocytes remained virtually unchanged (Fig. 3). When quantitated, the coefficient of osmotic water permeability (P_f) of the oocytes expressing CHIP28 rose 20-fold (to ~200 cm/s × 10^{-4}). Similar to water channels in native membranes, the Arrhenius activation energy was low, $E_a < 3$ kcal/mol (equivalent to diffusion of water in bulk solution); this was distinct from that of water-injected control oocytes, $E_a \gg 10$ kcal/mol. Also notable was the inhibition by 1 m*M* $HgCl_2$, which was reversed by the reducing

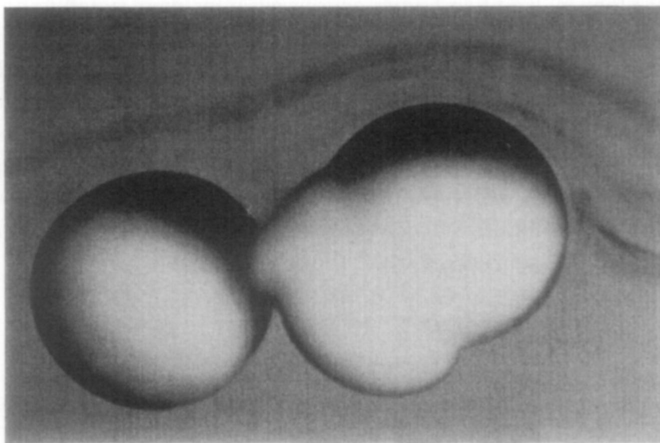


FIGURE 3 Osmotic swelling and rupture of *X. laevis* oocyte expressing AQP1 protein (*right*) vs water-injected control oocyte (*left*). Reproduced with permission from *Science* (Preston *et al.*, 1992).

agent 2-mercaptoethanol. The possibility that this permeability represented a non-specific leak was ruled out, because oocytes retained their normal resting potentials and failed to exhibit increased membrane currents. The lack of ion conductance by AQP1 was disputed by a single group of investigators who reported Forskolin-induced cation permeation by AQP1 expressed in oocytes (Yool *et al.,* 1996); however, this was not reproduced by other laboratories (Agre *et al.,* 1997). More recently, the originators of the AQP1–ion channel hypothesis have reported a cGMP gated conductance when AQP1 is expressed under special circumstances (Anthony *et al.,* 2000), an interesting observation that awaits confirmation. Other permeability issues are also still unresolved, including the interesting possibility that AQP1 is permeated by CO_2 or other gases (Nakhoul *et al.,* 1998; Yang *et al.,* 2000).

2. Reconstitution into Membranes

The initial oocyte expression studies were viewed as strong evidence that the CHIP28 protein is a membrane water channel, but the possibility remained that it could be an activator of endogenous water channels. Thus, highly purified protein was reconstituted at different concentrations into membrane proteoliposomes (Fig. 4), which were used for biophysical analysis of membrane water permeability (Zeidel *et al.,* 1992, 1994). Purified CHIP28 protein was reconstituted with pure phospholipid (protein:lipid ranging from 1:10 to 1:100), yielding small unilamellar vesicles of ~100 nm in diameter. These CHIP28 proteoliposomes were loaded with carboxyfluorescein and analyzed by stopped-flow transfer from iso-osmolar buffer to hyperosmolar solution, resulting in shrinkage of the vesicles with a

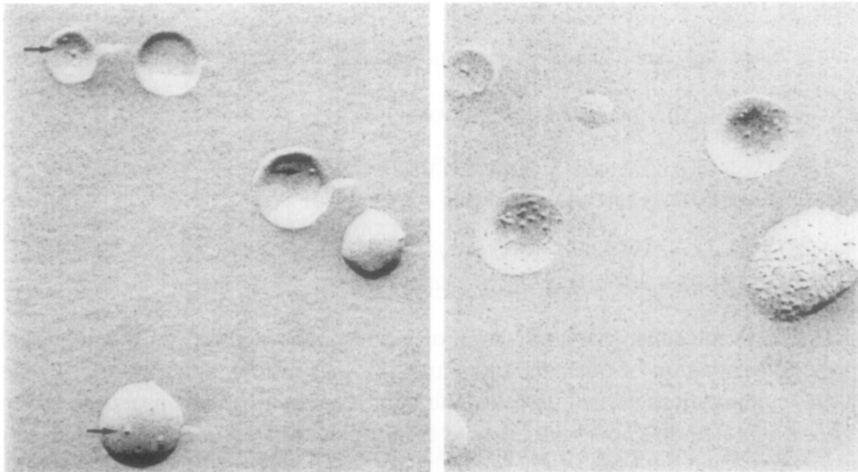


FIGURE 4 AQP1 reconstituted into proteoliposomes. Freeze fracture electron micrograph of membranes reconstituted with lower (left) or higher (right) concentrations of AQP1 protein. Reproduced with permission from *Biochemistry* (Zeidel *et al.,* 1994).

timescale of milliseconds. By comparisons with control liposomes (reconstituted membrane vesicles lacking CHIP28), the background water permeability was determined. The CHIP28 proteoliposomes exhibited up to a 50-fold increase in water permeability with low Arrhenius activation energy and mercurial inhibition. The unit permeability was calculated to be $\sim 3 \times 10^9$ water molecules per subunit per second. Moreover, permeability to H^+ and urea was not detected. Proteolytic removal of the 4-kDa C-terminal peptide did not diminish water permeability. Together these studies confirmed that CHIP28 is a constitutively active membrane water channel and quantitatively sufficient to explain the water permeability of red blood cells. Using partially purified CHIP28 protein, similar studies were undertaken by other investigators with similar interpretations (van Hoek and Verkman, 1992).

C. Aquaporins, a New Name for an Ancient Protein Family

As part of the Human Genome Project, the Genome Nomenclature Committee was charged with the issue of classifying genes that had been given different names by different laboratories. Lack of uniform nomenclature is a problem that has been present since ancient times. In *Genesis*, the effort to build a tower with its top in the heavens suddenly stopped when God caused multiple languages to be spoken by the workers, hence the Tower of Babel. Even in modern times, workers are often reluctant to accept and use nomenclature proposed by competing investigators, so the Human Genome Organization appointed a nomenclature committee to assign gene symbols. Several observations from the Human Genome Nomenclature Committee seem to reflect basic human nature:

> It is always the people with the worst data that make the most fuss about the gene symbol.
>
> —Phyllis McAlpine, University of Winnipeg

> People will accept any gene symbol, if it is No. 1.
>
> —Sue Povey, University of London

> The use of aliases will continue, but only by the individuals who invented them.
>
> —Alan Scott, Johns Hopkins University

The first molecular characterization of a molecular water channel quickly led to the identification of other mammalian homologs and plant homologs. To aid in recognizing these related proteins, the name "aquaporin" was proposed (Agre *et al.*, 1993). This has now been adopted as the name for all sequence-related water transporters, and the gene symbol *AQP* has been adopted by the Human Genome Nomenclature Committee (Agre, 1997). CHIP28 is now officially referred to as AQP1, and this nomenclature is used throughout the remainder of this volume.

D. Structure of AQP1 Protein

Determination of protein structures is a major step in the development of pharmacological agents. Soon after its discovery, the structure of AQP1 was pursued by multiple scientific groups.

1. Structure Deduced from Sequence

Hydropathy analysis of the deduced amino acid sequence of lens MIP protein predicted it to be an integral membrane protein with six bilayer-spanning domains (Gorin *et al.*, 1984). Closer inspection revealed two tandem repeats (Figs. 2 and 5), each containing three bilayer-spanning domains with two perfectly conserved motifs in the connecting loops, Asn-Pro-Ala (NPA) (Reizer *et al.*, 1993; Wistow *et al.*, 1991). The demonstration of water channel function made it possible to engineer recombinants with the goal of establishing the sites of mercurial inhibition and the membrane topology of biologically active recombinants.

Mercurials are known to react with free sulfhydryls, and the primary sequence of AQP1 contains four cysteines. In individual recombinants, each cysteine was replaced by a serine, an amino acid with the same structure except where the sulfur atom is replaced by an oxygen (Preston *et al.*, 1993). These studies implicated the residue Cys 189 in loop E as the site of mercurial inhibition and suggested that this domain may form a critical narrowing in the aqueous pore. In subsequent studies, the mercury-insensitive mutant Cys 189 Ser was further mutated by introducing a cysteine at the corresponding residue in loop B, Ala 73 Cys. This restored mercurial sensitivity, indicating that loops B and E are both functionally important. Recombinants were prepared in which monomers, containing or lacking the Hg^{2+} inhibitory site, were linked in tandem as 55-kDa dimers. Analyses were undertaken of the dimers as well as immunoprecipitation of full-length and truncated forms of the AQP1 monomers. Together, all of these studies indicated that each of the four monomers in the AQP1 tetramer carries an individual aqueous pore (Jung *et al.*, 1994a).

*Bam*HI restriction sites were cloned into the AQP1 cDNA at different sites. DNA encoding a 31-residue peptide epitope from the coronavirus E1 glycoprotein was then inserted at these different sites, and the water transport was assessed by expression in oocytes (Preston *et al.*, 1993). This epitope was helpful, because specific antibodies were available and because the epitope contains proteolytic cleavage sites, whereas the intact AQP1 protein does not.

Insertions into loops B or E usually caused loss of function when the recombinants were expressed in oocytes (Jung *et al.*, 1994a; Preston *et al.*, 1993). Nevertheless, insertions into the N or C termini or connecting loops A, C, or D encoded functional molecules which were then evaluated to see whether the E1 epitope was at the extracellular surface or the cytoplasmic surface of the oocyte plasma membrane. By antibody binding or by selective proteolysis, the epitope was

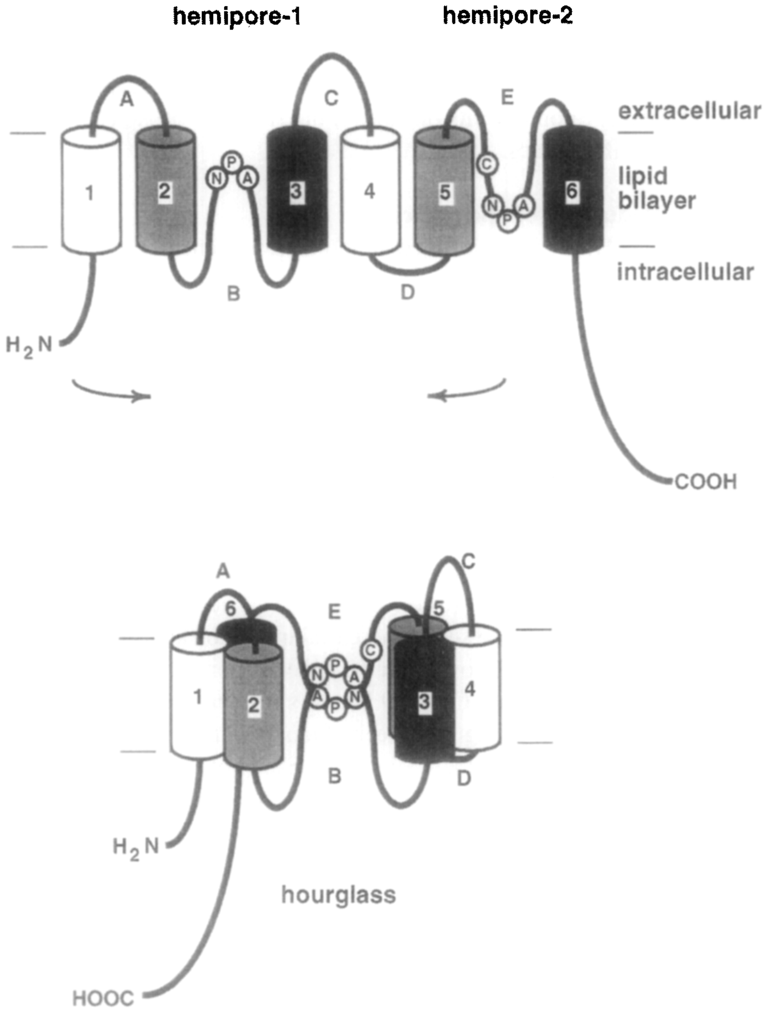


FIGURE 5 Schematic diagram of AQP1 monomer showing hourglass structure. (*Top*) Two repeats of three bilayer-spanning domains are oriented in obverse symmetry. Loops B and E are believed to fold back into the membrane. (*Bottom*) When folded together, the repeats form a single aqueous pathway through the membrane. Reproduced with permission from *The Journal of Biological Chemistry* (Jung *et al.,* 1994a).

confirmed to reside at the previously predicted positions. Of greatest importance was the location of loop C at the extracellular side of the membrane. This placed a constraint on the AQP1 topology, indicating that the two repeats must be obversely symmetric with a structure resembling the ancient hourglass (Fig. 5). Another

group of investigators approached the topology problem by preparing truncated molecules (Skach *et al.,* 1994). They initially concluded that the water channel is composed of four transmembrane domains. It is now believed, however, that this may represent an intermediate state (see Chapter 5). Thus, the hourglass model was generally believed to be the most likely structure (Jung *et al.,* 1994a).

2. Protein Structural Determinations

At the same time reconstitution of purified AQP1 protein was being functionally characterized (see above), the protein was being analyzed for structure. The hydrodynamic properties of the purified AQP1 protein revealed it to be a homotetramer composed of four 28-kDa subunits embedded in the membrane with only a 4-kDa C terminus extending into the cytoplasm (Smith and Agre, 1991). Cultured cells transfected with the AQP1 cDNA as well as proteoliposomes were shown by negative staining electron microscopy to have a multisubunit structure interpreted as being a tetramer (Verbavatz *et al.,* 1993).

Given the serious difficulties associated with crystallizing integral membrane proteins for X-ray diffraction crystallography, the structure of the AQP1 protein was undertaken by cryo-electron microscopy of membrane crystals. Reconstituting purified AQP1 protein at high concentrations relative to the concentration of phospholipid (approximately 1:1) yielded large, highly ordered membrane sheets and resealed membrane vesicles of ~300 μm in diameter. Measuring the water permeability of the membrane vesicles confirmed that the reconstituted protein was 100% biologically active (Walz *et al.,* 1994). A series of studies revealed the 2D structure at increasing resolution, and by studying the membrane crystals at tilts of up to 60°, the 3D structure was established at 6Å (Cheng *et al.,* 1997; Li *et al.,* 1997; Walz *et al.,* 1997). The temperature of the electron diffractions is critical to the measurements, and the use of a special electron microscope with a liquid-helium-cooled stage, has now established the 3D structure at 3.8 Å. Short pore helices are now identified after the NPA motifs in loops B and E, and protrusions representing side chains are visualized (Mitsuoka *et al.,* 1999). Merging these determinations with model building has yielded the structure at the atomic level (Murata *et al.,* 2000). A narrow aqueous pore through the center of AQP1 is flanked by critical residues (Fig. 6).

The structural studies have concentrated on the membrane domains, because this part of the molecule confers water transport (Zeidel *et al.,* 1994). The importance of the N and C termini is not understood, and these domains are least well conserved when species orthologs and other aquaporin paralogs are compared. The N and C termini are believed to be important in membrane targeting, but recent work suggests that in some tissues, AQP1 may associate with other proteins by being part of a large complex associated through a PDZ binding motif (Cowan *et al.,* 2000). Certainly this area of research awaits further investigation.

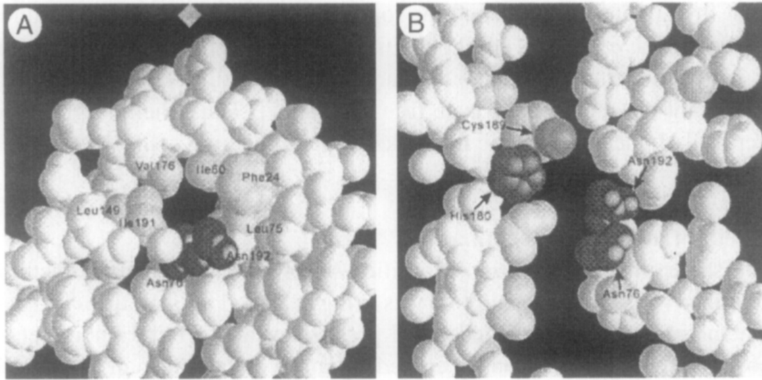


FIGURE 6 Space-filled diagrams of the aqueous pore in AQP1. A) Section through AQP1 monomer parallel to the plane of the membrane showing functionally important hydrophilic residues (darkly shaded–Asn76 and Asn192). B) Section showing side view of narrow aqueous channel flanked by Hg^{++} inhibitory site (Cys189). Reproduced with permission from *Nature* (Murata *et al.*, 2000).

E. Tissue Distribution

Prior to cloning of the cDNA encoding AQP1, it had been recognized that multiple tissues exhibit high levels of water permeability, and these tissues were believed to contain molecular water channels. With the development of high-affinity antibodies specific for the N and C termini of AQP1, it became possible to precisely locate the cellular and subcellular sites where the protein is expressed. These studies provided great insight into the physiological and pathophysiological roles for the AQP1 protein.

1. AQP1 in Kidney

Beginning with Homer Smith in the 1930s, transport physiologists have given the kidney more attention than any other tissue. Despite its great importance, Smith recognized the limitations of kidney research:

> The history of renal physiology has erred, more often than not, by attempts at oversimplification. The problems of water and salt excretion appear to be extremely complex, and especially liable to this danger.
>
> —Homer W. Smith, 1937, *The Physiology of the Kidney*

The kidney is a major site where nitrogenous wastes are excreted, pH is adjusted, and body water balance is achieved. Each kidney contains roughly one million individual nephron units: glomerulus, proximal convoluted and straight tubules, descending thin limbs of Henle's loop, thin and thick ascending limbs, and connecting tubules that join multiple nephrons to the collecting ducts. The average adult human generates almost 200 liters of glomerular filtrate daily, but

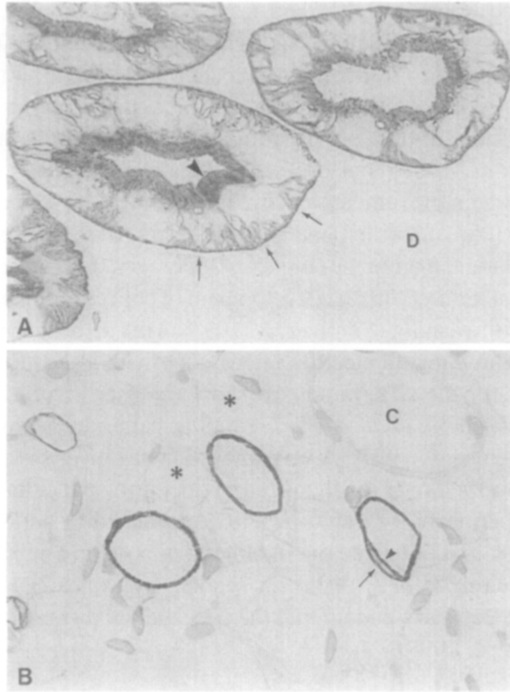
almost 90% of the water is reabsorbed in the proximal tubules and descending thin limbs. These tissues are known to be highly permeable to water, and water channels were predicted to reside at these locations. Using affinity-purified polyclonal antibodies, AQP1 was identified at the apical brush border and at the basal and lateral membranes of both proximal tubules and descending thin limbs of rat kidney (Denker *et al.,* 1988; Nielsen *et al.,* 1993a) (Fig. 7A,B).

Transcellular water movements occur through AQP1. Water enters through AQP1 in the apical brush border, and water is released into the interstitium from AQP1 in the basolateral membrane (Fig. 7C). Moreover, the appearance of this protein coincides in rat kidney with the development of concentrating mechanisms that occur at the time of weaning (Smith *et al.,* 1993). This distribution was confirmed in rat kidney by immunogold electron microscopy with a second affinity-purified antibody specific for the N terminus of AQP1 (Nielsen *et al.,* 1993a) and with whole antiserum (Sabolic *et al.,* 1992). A similar pattern was observed in human kidney (Maunsbach *et al.,* 1997). A very important negative was established when AQP1 was not observed over renal collecting duct principal cells (Fig. 7A,B), the site where water permeability was known to be regulated by antidiuretic hormone (vasopressin). This first indicated the likelihood of a second member of the aquaporin family (Nielsen *et al.,* 1993a). A second area of AQP1 expression was observed over the capillary endothelia, the descending vasa recta (Nielsen *et al.,* 1995b; Pallone *et al.,* 1997).

2. AQP1 in Other Tissues

In situ hybridizations indicated that AQP1 is expressed in complex patterns (Bondy *et al.,* 1993). AQP1 mRNA transiently appears in some tissues near the time of birth (e.g., corneal endothelium, periosteum, and heart). AQP1 mRNA first appears in other tissues around the time of birth and throughout adult life (e.g., kidney, lung, and blood-forming tissues). AQP1 mRNA was observed in still other tissues throughout fetal and postnatal life (e.g., choroid plexus). The affinity-purified antibodies were used to establish that AQP1 protein appeared in each of these tissues (Nielsen *et al.,* 1993b).

Of particular importance, AQP1 was found to be abundant in capillary endothelia in many distributions throughout the body where the protein was shown by light microscopy and immunogold electron microscopy to reside in both lumenal and ablumenal membranes. In lung, this was especially prominent in the capillary endothelia in peribronchiolar regions, indicating a special need for this protein in the reabsorption of fluid just after birth or in settings of interstitial edema (King *et al.,* 1996; Umenishi *et al.,* 1996). Premature human infants suffer significant morbidity due to immature lung, and glucocorticoid therapy is known to improve lung function. A classical glucorticoid response element is located in the proximal promoter region of the human *AQP1* gene (Moon *et al.,* 1997), and treatment of pregnant rats with glucocorticoids results in a 5- to 10-fold boost in AQP1 expression in the fetal lungs. Other sites where AQP1 is expressed include liver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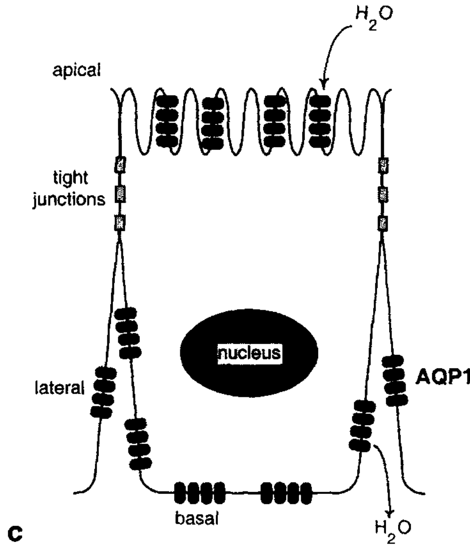
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cholangiocytes (Roberts *et al.*, 1994) and fibroblasts beneath airway epithelium (King *et al.*, 1996, 1997; Nielsen *et al.*, 1997a). The protein is also expressed in choroid plexus, where it may participate in generation of cerebrospinal fluid; in nonpigment epithelia in eye, where it may participate in formation of aqueous humor; and in corneal endothelium and lens epithelium (Nielsen *et al.*, 1993b).

F. AQP1-Null Humans

The presence of AQP1 in diverse tissues suggested a fundamental role for this protein; however, the clinical consequences of AQP1 deficiency were initially unclear.

Polymorphisms in red cell membrane proteins are known to be the molecular basis of blood group antigens. Although hundreds of different human red cell antigens have been described serologically, these belong to approximately 24 different blood groups, with each group representing a single genetic locus (Agre and Cartron, 1992). Previous discovery of some blood groups occurred when individuals totally lacking a certain antigen were sensitized with the blood of an individual who expresses the antigen. Although sensitization may occur after blood transfusions, relatively few individuals ever receive blood transfusions. Sensitization also occurs when maternal and fetal blood mixing occurs during birth. Because of potentially severe clinical manifestations, this is best recognized in the setting of maternal–fetal Rh incompatibilities; however, other blood group antigens can also lead to the appearance of circulating antibodies.

By classic linkage analyses, the human Colton blood group antigens were linked to the short arm of human chromosome 7 (Zelinkski *et al.*, 1990). Demonstration of the *AQP1* gene locus at the short arm of human chromosome 7 provided a strong clue that AQP1 protein is the structural basis of Co antigens (personal discussions with Marion Reid and Colvin Redman, New York Blood Center, 1993). Human Co antigens are generally not clinically important; 99.8% of the population is Co(a+), and ~8% is Co(b+). The International Blood Group Reference Laboratory in Bristol, United Kingdom, maintains a registry of all rare blood types, and DNA from peripheral blood leukocytes was obtained from multiple individuals with

FIGURE 7 AQP1 in kidney. (A) Immunohistochemical staining of AQP1 in rat renal cortex. Specific staining reveals abundant AQP1 in apical brush border (arrowhead), basal, and lateral membrane (small arrows) in proximal convoluted tubule. Distal convoluted tubules (D) fails to label. (B) Immunohistochemistry of AQP1 in apical and basolateral membrane in descending thin limb of Henle's loop. Ascending thin limbs (*) and collecting ducts (C) fail to label. Reproduced with permission from *The Journal of Cell Biology* (Nielsen *et al.*, 1993a). (*Bottom*) Schematic model showing transcellular water permeation of proximal tubule through AQP1. Reproduced with permission from *Kidney International* (Nielsen and Agre, 1995).

each phenotype. Polymerase chain amplifications of genomic DNA revealed that a polymorphism at residue 45 of AQP1 is the basis of the blood group. Ala 45 occurs in Co(a+), and Val 45 occurs in Co(b+) (Fig. 2); this specificity was confirmed by multiple immunoprecipitations and immunoblotting (Smith *et al.,* 1994).

Worldwide referencing has yielded only six individuals who lack the Co blood group antigens, and we were able to obtain blood and urine from three unrelated Co-null individuals (Preston *et al.,* 1994b). Immunoblotting revealed a total lack of AQP1 protein in the red cells from Probands 1 and 2, and only extremely low levels of the AQP1 protein in red cells from Proband 3. This was accompanied by a marked diminution in the red cell osmotic water permeability (Mathai *et al.,* 1996; Preston *et al.,* 1994b). Immunoblotting of urine sediment confirmed the lack of AQP1 protein in renal brush border membranes. Genomic DNA analyses demonstrated disruptions in *AQP1* (Preston *et al.,* 1994b). Proband 1 was homozygous for complete deletion of the first exon of *AQP1,* which normally encodes half of the AQP1 protein. Proband 2 was homozygous for a frameshift in the first exon. Proband 3 is homozygous for a missense mutation at the end of the first transmembrane helix where Pro 38 is replaced by Leu. Presumably this individual expresses an unstable form of AQP1, because when the P38L protein is expressed in oocytes, it is degraded.

All three Co-null individuals were identified because they developed anti-Co antibodies after pregnancy; the clinical courses, however, were not identical. Proband 2 successfully bore four children without incident. In contrast, the third pregnancy of Proband 1 was complicated by a life-threatening fetal hemolytic syndrome, which required intrauterine transfusions of the fetus with Co-null red cells from the mother. Because of persisting anti-Co antibodies, all three probands are at risk for hemolytic reactions if transfusions should ever be given. Thus, units of their own blood have been cryopreserved at nearby hospitals should emergency transfusions ever be needed.

Surprisingly, none of these Co-null individuals (referred to here as "AQP1 null") was aware of any other significant clinical difficulties. To ascertain whether subclinical deficiencies may exist, careful clinical evaluations were undertaken in the Clinical Research Unit at the Johns Hopkins Hospital. Because they totally lack AQP1 protein, Probands 1 and 2 were studied. Baseline evaluations of both were entirely normal, but after 24-h fluid deprivation, neither could concentrate her urine above 450 mos*M*, whereas normal individuals all concentrated their urine to $\sim$1000 mos*M* after only 8 h. Likewise, hyperosmolar saline infusions failed to raise the urinary osmolality, even though it caused a significant rise in serum osmolality. Although the individuals do not suffer as long as water is available, it is easy to see why AQP1 deficiency would be problematic for individuals with limited access to water.

Other evaluations of the AQP1-null individuals were undertaken, including high-resolution computer axial tomography of lung before and after intravenous fluid

challenge. Normal individuals experience a 2- to 3-fold increase in the thickness of airways after infusions, whereas neither of the two AQP1-null individuals exhibited a change in airway thickness. Thus, rate of fluid exchange between blood vessels and peribronchiolar interstitium is reduced when AQP1 is absent. Although no evidence suggests that AQP1-null families have increased problems with clearing perinatal lung edema, this remains a distinct possibility. By traditional gene disruption techniques, mice lacking AQP1 protein have been produced. Similar to AQP1-null humans, these mice exhibit multiple subtle defects (see Chapter 5).

III. OTHER MAMMALIAN AQUAPORINS

Discovery of AQP1 rapidly led to homology cloning efforts in multiple laboratories. Curiously, each new homolog was simultaneously reported by different research groups. This mini-industry has identified 10 mammalian homologs, and several have been partially characterized (Fig. 8).

A. Orthodox Aquaporins

The first characterized member of the family, AQP1, is freely permeated by water but not by other solutes. Several other members of the family were also found to be highly water selective.

1. AQP2, the Vasopressin-Regulated Water Channel

First attention was directed toward the kidney, where a cDNA encoding the anticipated collecting duct homolog was amplified using degenerate primers designed from the sequence of AQP1 (Fushimi *et al.,* 1993). A series of reports defined this protein in various physiologic and clinical states (see Chapters 3, 4, and 6). Although it was predicted to be the vasopressin-regulated water channel, formal demonstration was achieved by an elegant report in which collecting ducts were isolated from rat kidney and examined for water permeability and the distribution of AQP2 was established by immunogold electron microscopy (Fig. 9A) (Nielsen and Agre, 1995). In summary, AQP2 is rapidly targeted to the plasma membrane and reinternalized, thereby confirming the "shuttle hypothesis" and demonstrating a rapid form of regulation (Fig. 9). In addition, chronic thirsting was shown to lead to an increase in AQP2 biogenesis.

Large interest in AQP2 resulted in the identification of humans with mutations in the gene encoding AQP2 and a severe clinical phenotype (Deen *et al.,* 1994). Vasopressin-deficient individuals are unable to concentrate their urine and suffer from diabetes insipidus (DI). Subsequently, a group of patients with

Aquaglyceroporins
(Cocktail Set)

hAQP7L
rAQP7
hAQP3
rAQP9
glpF
hAQP1
rAQP6
rAQP8
hAQP5
hAQP2
aqpZ
hAQP0
(MIP)
hAQP4

Aquaporins
(Orthodox Set)

```
AqpZ --------------------MFRKLAAECFGTFWLVFGGCGSAVLAAGFP-----ELGIG  35
AQP1 ---------MASEFKK--KLFWRAVVAEFLATTLFVFISIG-SALGFKYPVGNNQTAVQD  48
AQP3 MGRQKELVSRCGEMLHIRYRLLRQALAECLGTLILVMFGCG-SVAQVVLSR----GTHGG  55
GlpF -------------MSQT-STLKGQCIAEFLGTGLLIFFGVG-CVAALKVA-----GASFG  40

AqpZ FAGVALAFGLTVLTMAFAVGHISGGHFNPAVTIGLWAGGRFPAKEVVGYVIAQVVGGIVA  95
AQP1 NVKVSLAFGLSIATLAQSVGHISGAHLNPAVTLGLLLSCQISIFRALMYIIAQCVGAIVA 108
AQP3 FLTINLAFGFAVTLGILIAGQVSGAHLNPAVTFAMSFLARDPWIKLPIYTLAQTLGAFLG 115
GlpF QWEISVIWGLGVAMAIYLTAGVSGAHLNPAVTIALWLFACFDKRKVIPFIVSQVAGAFCA 100

AqpZ AALVYLIASGKT-GFD---AAASG----FASNGYGEHSPGGYS-MLSALVVELVLSAGFL 145
AQP1 TAILSGITSS-----------LTGNS--LGRNDLADGVNSGQ-----GLGIEIIGTLQLV 150
AQP3 AGIVFGLYYDAIWHFADNQLFVSGPN---GTAGIFATYPSGHLDMINGFFDQFIGTASLI 171
GlpF AALVYGLYYNLFFDFEQTHHIVRGSVESVDLAGTFSTYPNPHINFVQAFAVEMVITAILM 159

AqpZ LVIHGATDKFA----PAG-FAPIAIGLALTLIHLISIPVTNTSVNPARSTAV---AIFQG 197
AQP1 LCVLATTDRRRRD--LGG-SAPLAIGLSVALGHLLAIDYTGCGINPARSFGS---AVITH 204
AQP3 VCVLAIVDPYNNPG-PRG-LEAFTVGLVVLVIGTSMGFNSGYAVNPARDFGPRLFTALAG 229
GlpF GLILALTDDGN--GVPRGPLAPLLIGLLIAVIGASMGPLTGFAMNPARDFGPKVFAWLAG 217

AqpZ G----WALE----QLWFFWVVPIVGGIIGGLIYRTLL------------------EKRD 230
AQP1 N----FS-N-----HWIFWVGPFIGGALAVLIYDFILAPRSSDLTDRVKVWTSGQVEEYD 254
AQP3 WGSAVFTTGQ--HWWWVPIVSPLLGSIAGVFVYQLMIGCHLE---------QPPPSNEEEN 279
GlpF WGNVAFTGGRDIPYFLVPLFGPIVGAIVGAFAYRKLIGRHLP---------CDICVVEEKE 269

AqpZ ---------------                                             231
AQP1 LDADDINSRVEMKPK                                             269
AQP3 VKLAHVKHKEQI---                                             292
GlpF TTTP-SEQKASL---                                             281
```

an X-linked disorder in which patients' kidneys fail to respond to vasopressin (nephrogenic diabetes insipidus, NDI) was identified, and several of these kindreds were found to carry mutations in the gene encoding the vasopressin V2 receptor on the X chromosome (reviewed by Bichet, 1998). Once the AQP2 water channel was identified, genomic DNA from NDI kindreds lacking V2 receptor mutations were found to contain mutations in *AQP2* causing recessive (Van Lieburg *et al.*, 1994) or dominantly inherited disorders (Mulders *et al.*, 1998).

Although studies thus far have been restricted to rodent models, several common clinical disorders of water balance are now believed to reflect secondary abnormalities in AQP2 expression. These provocative studies suggest that the human counterparts of these disorders will be linked to AQP2 expression. Important advances include the recognition that expression of AQP2 protein is reduced after lithium administration, a commonly used agent in the treatment of manic depressive disease that is known to produce polyuria (Marples *et al.*, 1995). Polyuria is a frequent side effect after removal of urinary obstruction (Frokiaer *et al.*, 1996), in diuretic-induced hypokalemia (Marples *et al.*, 1996), and in nocturnal enuresis (Frokiaer and Nielsen, 1997); AQP2 down-regulation has been described in each of these settings. Overexpression of AQP2 may contribute to the excessive water retention that frequently complicates congestive heart failure (Nielsen *et al.*, 1997c; Xu *et al.*, 1997). Most recently, overexpression of AQP2 has been implicated in the fluid retention during pregnancy (Ohara *et al.*, 1998), which may precipitate eclampsia.

2. AQP0 (MIP) from Lens Fiber Cells

Although the function of this protein is still unsettled, it was the first member of the family to be isolated and cloned (Gorin *et al.*, 1984). When expressed in oocytes, AQP0 confers only a modest increase in membrane permeability (Chandy *et al.*, 1997; Kushmerick *et al.*, 1995; Mulders *et al.*, 1995); however, it was recently noted that water permeability is elevated under slightly acidic conditions, pH 6 (Nemeth-Cahalan and Hall, 2000). Although the protein was reported to conduct ions when reconstituted into planar lipid bilayers (Ehring *et al.*, 1990), increased membrane currents were not detected when AQP0 is expressed in oocytes (Mulders *et al.*, 1995).

AQP0 protein is expressed only in fiber cells of lens, a site not known to have large levels of fluid transport, and a secondary structural role as a cell-to-cell

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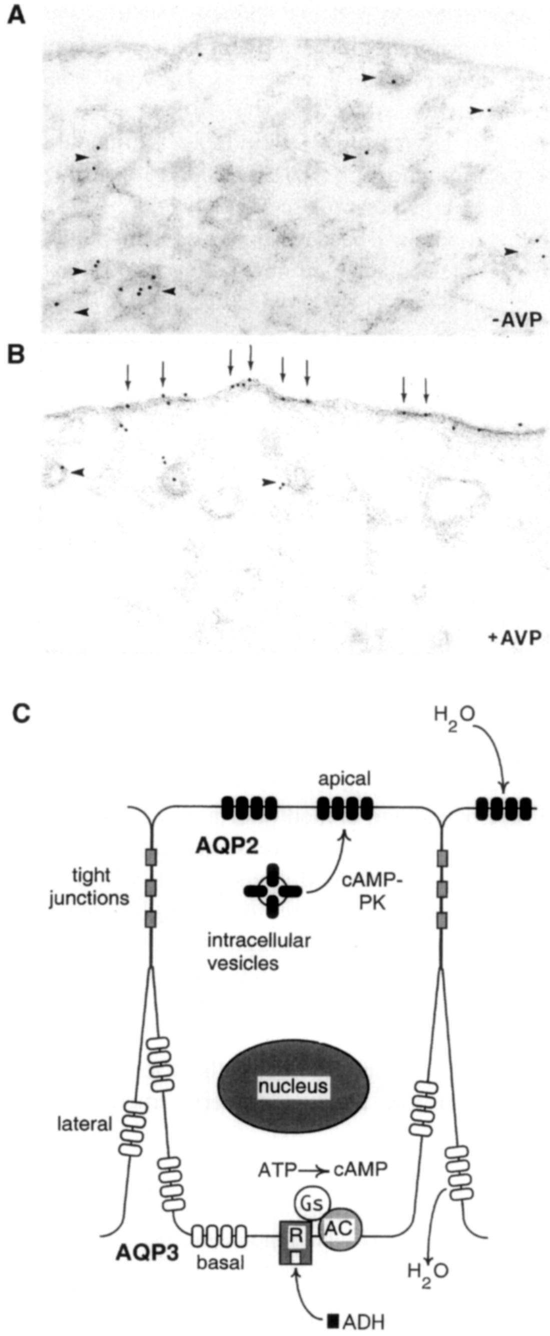
FIGURE 8 Phylogenetic and sequence analysis of mammalian and *Escherichia coli* aquaporin homologs. (*Top*) Mammalian aquaporins (*shaded*) and aquaglyceroporins (*unshaded*). Reproduced with permission from *The Journal of Biological Chemistry* (Agre *et al.*, 1998a). (*Bottom*) Sequence alignment of mammalian AQP1 and AQP3 with *E. coli* AqpZ and GlpF. Reproduced with permission from *The Annual Review of Biochemistry* (Borgnia *et al.*, 1999).


A
-AVP
B
+AVP
C
H2O
apical
AQP2
tight junctions
cAMP-PK
intracellular vesicles
nucleus
lateral
ATP→cAMP
Gs
AC
R
AQP3
basal
H2O
ADH

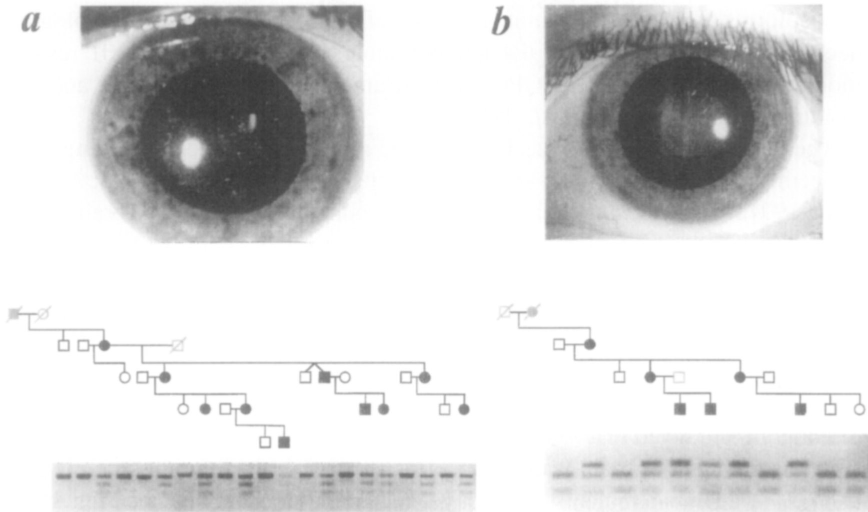


FIGURE 10 Cataracts in patients from two unrelated families with mutations in the gene encoding AQP0. (*a*) Lens examination of patient with T138R mutation showing multiple opacities throughout. (*b*) Lens examination of patient with E134G mutation showing single lamellar opacity. (*Bottom*) Restriction fragment analyses demonstrates cosegregation of patterns with cataract in both families (black symbols). Reproduced with permission from *Nature Genetics* (Berry *et al.*, 2000).

adhesion protein has been proposed (Fotiadis *et al.*, 2000). The protein is essential for lens homeostasis, because mice bearing mutations in the gene suffer from congenital cataracts (Shiels and Bassnett, 1996). This is now known to be responsible for some forms of congenital cataract in humans (Berry *et al.*, 2000; Francis *et al.*, 2000). Interestingly, in mice and humans, the cataracts are a dominant trait, consistent with a structural defect, and the different mutations cause clinically distinct forms of cataract (Fig. 10).

3. AQP4, the Brain Water Channel

The homolog was cloned from brain (Jung *et al.*, 1994b) and lung (Hasegawa *et al.*, 1994). Unlike most other aquaporins, AQP4 is not inhibited by mercurials. Also unlike other aquaporins, AQP4 has two translation initiation sites encoding

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FIGURE 9 AQP2 in principal cells of renal collecting duct. (A) Immunogold electron microscopy of rat collecting duct principal cell in unstimulated state. (B) Same after stimulation with vasopressin. Reproduced with permission from *Proceedings of the National Academy of Science USA* (Nielsen *et al.*, 1995d). (C) Schematic model for transcellular water movement through collecting duct principal cell as regulated by exocytosis of AQP2. Reproduced with permission from *Kidney International* (Nielsen and Agre, 1995).

two isoforms of 301 and 323 amino acids (Lu *et al.*, 1996). Although the biological need for this is uncertain, AQP4 homotetramers formed from the 301 residue monomers exist, as well as AQP4 heterotetramers containing both 301 and 323 residue monomers (Neely *et al.*, 1999).

The distribution of AQP4 has been defined in multiple tissues, including renal collecting duct principal cells and airway epithelia (Frigeri *et al.*, 1995; Nielsen *et al.*, 1997a; Terris *et al.*, 1995). AQP4 is most abundantly expressed in brain where it resides in the astroglial cells surrounding blood vessels (Fig. 11A) (Nagelhus *et al.*, 1998; Nielsen *et al.*, 1997b). Square arrays have been observed in the plasma membranes of astroglial and ependymal cells, but their molecular identities were uncertain. Similar arrays were observed in cultured cells transfected with *AQP4* (Yang *et al.*, 1996), and direct labeling of fracture replicas with anti-AQP4 established that square arrays contain AQP4 (Rash *et al.*, 1998) (Fig. 11B). This distribution predicted that AQP4 is involved in dissipation of brain edema or possibly disturbances of brain water homeostasis such as pseudotumor cerebrii (Lee *et al.*, 1997). Human mutants have not yet been identified, but mice bearing *AQP4* gene disruption were found to have improved survival in acute brain edema models (Manley *et al.*, 2000) (see Chapter 5). AQP4 is also expressed in fast twitch skeletal muscle fibers and is deficient in the mouse model of Duchenne's muscular dystrophy (Frigeri *et al.*, 1998). Regulation of AQP4 water permeability by protein kinase C was proposed after a study demonstrated that high concentrations of phorbol diesters partially reduce the water permeability of AQP4 oocytes (Han *et al.*, 1998); however, these agents are known to induce nonspecific perturbations of surface membrane.

4. AQP5 from Secretory Glands

This homolog was cloned from a submandibular gland cDNA library (Raina *et al.*, 1995). Immunohistochemical and immunogold electron microscopic studies also demonstrated the protein in the apical membranes of secretory glands, type I pneumocytes, and corneal epithelium (Hamann *et al.*, 1998; Nielsen *et al.*, 1997a). Although regulation of AQP5 has been proposed, no convincing evidence suggests that protein trafficking or gating occurs. The distribution implicates AQP5 in human dry disorders. Recent studies of lacrimal glands and salivary glands from patients with Sjögren's syndrome show abnormal trafficking of AQP5 (Steinfeld *et al.*, 2001; Tsubota *et al.*, 2001). In support of this, mice with disruptions in the gene encoding AQP5 were found to have reduced salivary gland secretion (Ma *et al.*, 1999).

B. Mammalian Aquaglyceroporins

AQP3, AQP7, AQP9, and possibly AQP8 constitute a subgroup of aquaporins with a broader permeation range that includes glycerol, hence the name

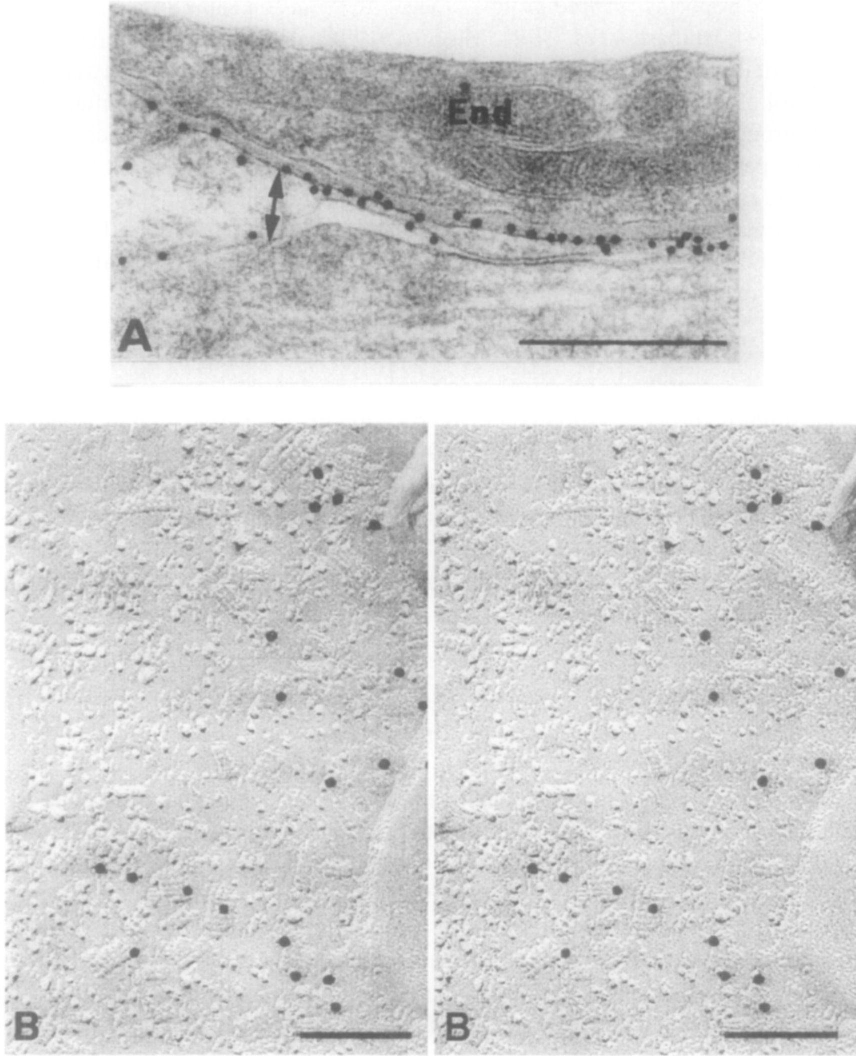

FIGURE 11 Immunoelectron micrographs of AQP4 in perivascular astroglial cells of rat brain. (A) Ultrathin section of rat cerebellum reacted with anti-AQP4. Reproduced with permission from *The Journal of Neuroscience* (Nielsen *et al.*, 1997b). (B) Stereoscopic view of end-feet in freeze fracture replica. Reproduced with permission from *The Proceedings of the National Academy of Science* (Rash *et al.*, 1998).

"aquaglyceroporins." Three groups cloned AQP3 at the same time (Echevarria *et al.*, 1994; Ishibashi *et al.*, 1994; Ma *et al.*, 1994). Although one group of scientists initially disputed the permeation by water, all agreed that the protein transports glycerol. Likewise, it seems most likely that the protein transports water and glycerol through the same pore (Kuwahara *et al.*, 1997) although evidence for two distinct pathways has been reported (Echevarria *et al.*, 1996). The distribution in multiple sites, including the basolateral domains of renal collecting duct principal cells, airway epithelia, and secretory glands, suggests several functions (Ecelbarger *et al.*, 1995; Frigeri *et al.*, 1995; Nielsen *et al.*, 1997a). Mice engineered with disruptions of the *AQP3* gene exhibit a nephrogenic diabetes insipidus phenotype (Chapter 5); however, human mutants have not yet been reported.

Curiously, AQP7, AQP8, and AQP9 were each simultaneously cloned by multiple groups. AQP7 is permeated by water and glycerol. The permeation of AQP8 and AQP9 is disputed, but strong evidence supports the permeation of AQP9 by a range of solutes (Tsukaguchi *et al.*, 1998). The subcellular distribution of these homologs is still uncertain and species differences have been reported. Although rigorous tissues and subcellular localization studies are not yet available, AQP7 is expressed in testis (Ishibashi *et al.*, 1997); AQP8 is expressed in pancreas and liver (Koyama *et al.*, 1997); and AQP9 is expressed in liver and leukocytes (Tsukaguchi *et al.*, 1998).

C. AQP6, a Very Strange Member of the Family

Another homolog (AQP6) was cloned from kidney but evoked marginal scientific interest because it was reported to bear only minimal water permeability, which was allegedly inhibited by Hg^{2+} (Ma *et al.*, 1995). The AQP6 protein was subsequently demonstrated to reside within intracellular vesicles in three kidney epithelia: glomerular podocytes and proximal straight tubules. The AQP6 protein is most abundantly expressed in the α-intercalated cells of collecting duct (Yasui *et al.*, 1999a). Note that these cells lie alongside the principal cells of collecting duct, where the AQP2 resides in intracellular vesicles traffic to the plasma membrane and are then reinternalized. In contrast, AQP6 appears exclusively in the membrane of intracellular vesicles alongside H^+-ATPase (Yasui *et al.*, 1999b). The α-intercalated cells are known to play an important function in acid–base metabolism by releasing acid into the urinary lumen.

The function of AQP6 was recently reevaluated by expression in oocytes. Surprisingly, the low level of water permeability was significantly increased by treatment with Hg^{2+} (Yasui *et al.*, 1999b), and two cysteine residues were demonstrated to be required for this effect (Fig. 12A). Moreover, the Hg^{2+}-treated AQP6 oocytes

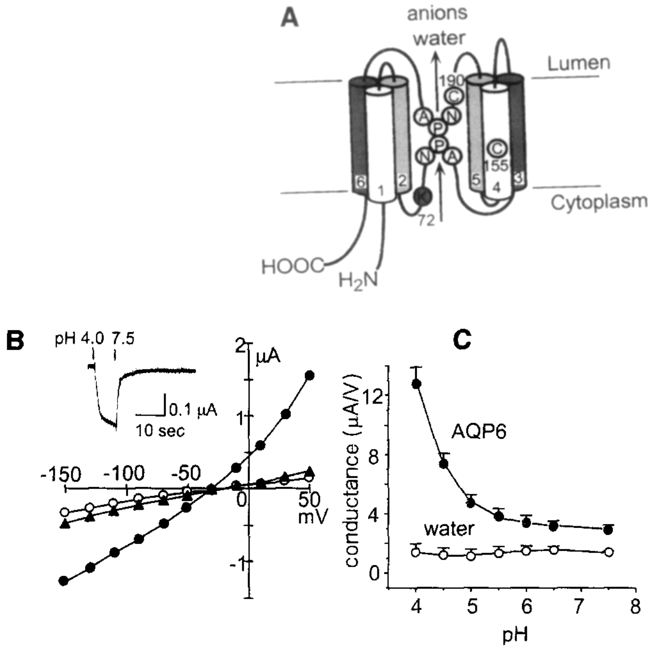

FIGURE 12 AQP6 model and electrophysiologic analyses in *X. laevis* oocytes. (A) Membrane topology of AQP6 showing positively charged residue (K72) at cytoplasmic mouth of pore, juxtaposed NPA motifs, and cysteines critical to Hg^{2+} activation (C155 and C190). (B) IV plot of AQP6 oocyte at pH 7.5 (open circles), after 1 min at pH 4.0 (solid circles), or 1 min after return to pH 7.5 (triangles). (C) Ion conductance of AQP6 oocytes and control water-injected oocytes measured at different pH. Reproduced with permission from *Nature* (Yasui *et al.*, 1999b).

were found to have a large membrane current that exhibited intermediate selectivity for anions. Because AQP6 is expressed in acid-secreting cells, it was questioned whether these effects may be triggered by low pH. A shift to pH 4.0 rapidly induced the ion permeation and swelling, which were rapidly reversed by return to pH 7.0 (Fig. 12B,C). AQP6 has several residues that are not present in other aquaporins. In particular, a charged residue Lys 72 lies at the cytoplasmic mouth of the pore (Fig. 12A), and mutagenesis to the negatively charged Glu led to loss of ion selectivity.

The presence of AQP6 in intracellular vesicles in α-intercalated cells suggests that the H^+-ATPase creates an intravesicular acid environment, opening AQP6 to maintain electroneutrality. The presence of AQP6 in proximal straight tubules suggests that the protein may participate in the acidification of endosomal vesicles. These features are distinct from those of all other members of the aquaporin family

and suggest that the biophysical properties of aquaporins may be much more complex than previously believed.

D. Multiple Aquaporins in a Tissue

The initial studies of AQP1 suggested that multiple homologs must exist to explain water permeation of tissues lacking the protein (Nielsen *et al.*, 1993a,b). Although not originally foreseen, multiple homologs have been identified in certain tissues. Indeed the assembly of several aquaporins creates a complex intracellular plumbing system. The biological need for multiple aquaporins seemingly reflects multiple needs: (1) the need for regulated and unregulated aquaporins; (2) the need for channels exclusively permeated by water or the need for channels permeated by water plus small solutes or even ions; and (3) the need for specific trafficking signals that target aquaporins to specific plasma membrane domains or intracellular sites in polarized epithelia.

As discussed earlier, the kidney contains at least six aquaporins in nonoverlapping distributions: AQP1, AQP2, AQP3, AQP4, AQP6, and AQP7. The collecting duct exhibits the greatest complexity, with principal cells containing AQP2 in intracellular vesicles and at the plasma membrane and AQP3 or AQP4 at the basolateral domains (Fig. 9C). The adjacent α-intercalated cells are only known to contain AQP6, whose primary function may be to provide anion transport.

The epithelia of lung tissues are also complex (Nielsen *et al.*, 1997a). Distal lung features AQP5 in the apical membrane of type I epithelia, a flattened cell type that provides most of the gas exchange surface area. Interestingly, no aquaporin has yet been identified on the basolateral membrane of these cells (Fig. 13A). In the airway epithelia, even more complexity is present with AQP3 in basal cells and AQP4 in the lateral membranes of surface epithelia. AQP1 is present in subepithelial fibroblasts and capillary endothelia (Fig. 13B). Secretory glands contain two different aquaporins in the basolateral membranes (AQP3 or AQP4) and AQP5 in the apical membrane (Fig. 13C).

The eye is also complex, with at least five different aquaporins present (Hamann *et al.*, 1998) (Fig. 13D). As noted previously, AQP1 exists in nonpigment ciliary epithelia, outflow tracks, lens epithelium, and corneal endothelium. It is noteworthy that AQP1-null humans are not known to exhibit ocular dysfunction. However, it is very possible that the protein is necessary during injury or other stress. AQP0 resides in lens, where its absence causes cataract. AQP3 resides in the conjunctivae, AQP5 in corneal epithelium and lacrimal glands, and AQP4 in retinal Müller cells. Presumed functions may be inferred from the sites of expression; however, demonstration of precise physiological or pathophysiological roles has not yet occurred.

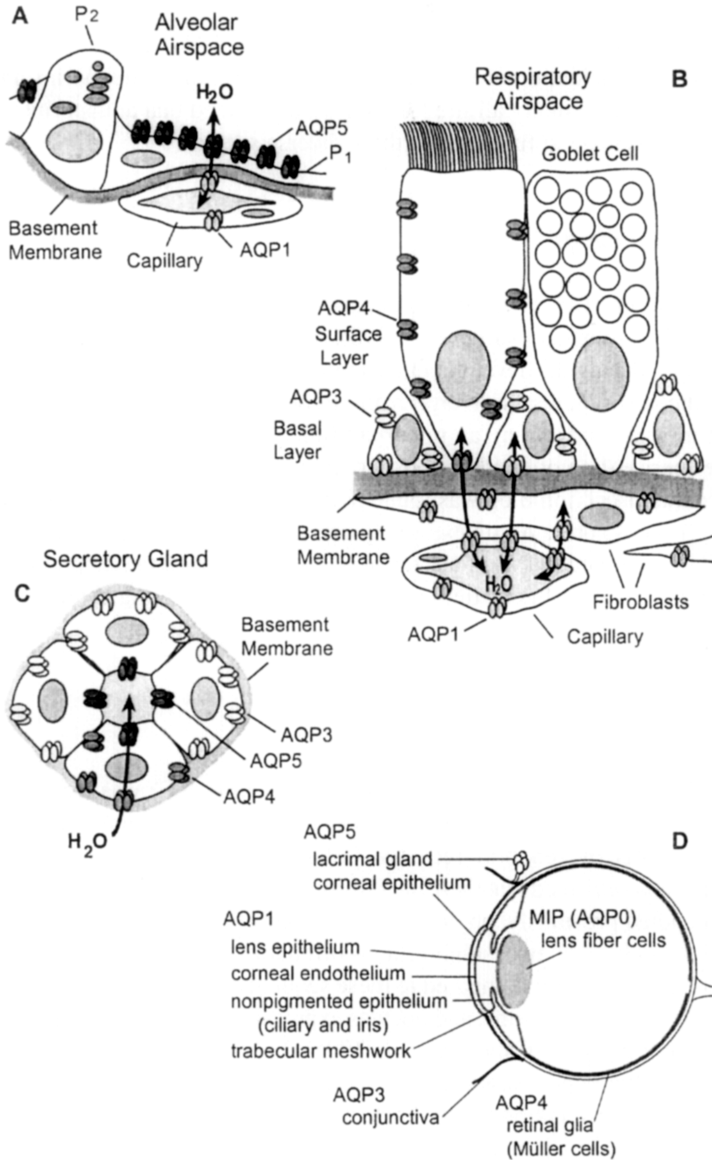

FIGURE 13 Schematic diagrams of multiple aquaporins in complex tissues of lung and eye. (A) Alveolus, (B) trachea, (C) secretory acinus, and (D) eye. Reproduced with permission from *The American Journal of Physiology* (Hamann *et al.*, 1998; Nielsen *et al.*, 1997a).

IV. NONMAMMALIAN HOMOLOGS

The recent elucidation of multiple genomes is revealing aquaporins and aquaglyceroporins in virtually all life forms. It is anticipated that aquaporins will play multiple physiological functions in these diverse species.

A. Insect Homologs

The big brain (Bib) *Drosophila* mutant was recognized before the function of AQP1 was established (Rao *et al.,* 1990). The mutant phenotype is a distinctive perturbation during the developmental switch that occurs when neural progenitors and epithelial progenitors diverge from a common cell type. Although the Bib protein must be performing an important function, no biophysical demonstration of water permeation or another transport event has yet been shown.

A second insect homolog was found in the sap-sucking insect *Cicadella viridis* (AQP_{CIC}). These proteins are extremely abundant in the filter plate of the midgut, where large amounts of fluid are taken in and transferred to Malpighian tubules (Beuron *et al.,* 1995). Interesting structural information is being obtained by studying this native protein.

B. Microbial Homologs

The *Escherichia coli* glycerol facilitator protein (GlpF) was discovered long before aquaporins were recognized (Heller *et al.,* 1980), but the second homolog (AqpZ) was discovered more recently (Calamita *et al.,* 1995). *E. coli* genome sequencing has confirmed that these are the only two aquaporin homologs in that bacteria. As noted previously, phylogenetic analysis of mammalian aquaporins splits the superfamily into two subfamilies—orthodox aquaporins and aquaglyceroporins (Fig. 8A). When compared to these sequences, AqpZ aligns with the mammalian orthodox aquaporins, and oocytes expressing this protein are permeated by water but not glycerol (Calamita *et al.,* 1995). In contrast, GlpF aligns with mammalian aquaglyceroporins, and oocytes expressing GlpF are permeated by glycerol, whereas water permeation was not detected (Maurel *et al.,* 1994).

Structural explanations for these functional differences are being sought. The primary amino acid sequences are highly homologous, yet a few sequence differences are present (Fig. 8B). Of note, AqpZ and orthodox aquaporins have a Ser following the second NPA motif in loop E (NPARS), whereas GlpF and aquaglyceroporins have an Asp at this site (NPARD) as well as a motif (GLYY) at the extracellular end of the third transmembrane domain. Electron microscopic studies of GlpF expressed in oocytes were interpreted as showing that the protein is a

monomer (Bron *et al.*, 1999), whereas electron crystallographic studies of purified, reconstituted GlpF at 7 Å showed a tetrameric assembly with a central hourglass geometry, suggesting a larger pore (Braun *et al.*, 2000). Moreover, two residues at the extracellular end of the sixth transmembrane domain have been reported to determine whether AQP_{CIC} transports water like an orthodox aquaporin (YW) or glycerol like an aquaglyceroporin (PL) (Lagrée *et al.*, 1998, 1999); however, preliminary studies from other laboratories have not confirmed the general significance of these observations.

Why a simple gram-negative organism would need two functionally related members of this protein family is puzzling. Presumably, the specificity of AqpZ for water but not glycerol and GlpF is important to the bacterium for glycerol transport. Nevertheless, null phenotypes are not strikingly obvious. AqpZ-null bacteria exhibit relatively less growth than the parent strain when cultured under conditions of maximal growth (Calamita *et al.*, 1998). The genomes of most bacteria contain only one member of the aquaporin family, and the functional importance is yet undefined.

Saccharomyces cerevisiae is a favorite organism for genetic manipulations, and the recent genome sequencing has revealed genes for four aquaporin homologs. Two encode proteins with sequences more closely related to aquaglyceroporins, of which the FPS1 homolog has been studied extensively (see Chapter 8). Two other sequences similar to orthodox aquaporins have been identified (Bonhivers *et al.*, 1998; Laizé *et al.*, 1999). In lab strains, *AQY1* encodes a protein that is not functional when expressed in oocytes. Presumably, this reflects negative pressure, because *AQY1* from wild-type strains encodes a functional water channel (Bonhivers *et al.*, 1998). The second gene, *AQY2*, is fragmented in the sequenced genome; however, other strains contain an open reading frame that is nonfunctional in oocytes (Laizé *et al.*, 2000). The physiological significance of these two yeast aquaporin proteins is still being sought, and it remains unknown whether microbial aquaporins are involved in invasiveness or other clinically important processes.

C. Plant Homologs

The plant kingdom contains a large and complex group of organisms that need careful water balance. Hydraulic engineering is involved in many physiological functions of plants. Not surprisingly, numerous aquaporins have been identified in plants, and each species may carry genes for several dozen aquaporin homologs. The functions of some of these proteins are being uncovered, and a complex and fascinating story is emerging (reviewed by Maurel, 1997) (see Chapter 7).

Briefly, three types of plant homologs have been identified: tonoplast intrinsic proteins (TIPs), plasma membrane intrinsic proteins (PIPs), and the symbiosome protein in the roots of legumes colonized with nitrogen-fixing bacteria (Nod26).

When expressed in oocytes, some of these proteins (γ-TIP) have been shown to transport water (Maurel *et al.*, 1993), whereas the plant homolog Nod26 has broader solute permeation (Dean *et al.*, 1999). Why many plant homologs do not exhibit any function when expressed in oocytes is not understood (M. J. Chrispeels, personal communication, 2000).

Some plant aquaporins have been linked to physiologically important processes. As noted previously, the existence of turgor-responsive genes in plants included a protein with a sequence related to AQP1 (Guerrero *et al.*, 1990), suggesting that some members of the plant aquaporins sense water deprivation. By genetically reducing expression of the PIP1b homolog in *Arabidopsis thaliana* (Kaldenhoff *et al.*, 1998), the plant was still able to maintain stem turgor by increasing the arborization of rootlets (Fig. 14). This provides an interesting lesson on the need for care during phenotype determinations, because the plants appeared similar above the surface. Curiously, another aquaporin homolog TobRB7 is up-regulated

FIGURE 14 Functional consequence of aquaporin PIP1b expression in *A. thaliana*. (*Left*) Plant overexpressing antisense RNA exhibits low PIP1b protein content and compensates with increased arborization of rootlets. (*Right*) Genetically unmodified plant. Reproduced with permission from *The Plant Journal* (Kaldenhoff *et al.*, 1998).

in the roots of tobacco plants during nematodal infestation, permitting the parasite to suck fluid from the host organism (Opperman *et al.,* 1994). To preserve the well-recognized benefits of genetic diversity (hybrid vigor), most plants defend against self-pollination. An interesting aquaporin mutant in the crucifer family eliminates a checkpoint, suggesting that water transport between stigma and pollen is involved in this process (Ikeda *et al.,* 1997). Other physiological and pathophysiological processes are being sought, and it is likely that aquaporins will attract the continued interest of plant physiologists.

V. PERSPECTIVE

Discovery of the aquaporins has dramatically changed the field of membrane water transport, and the impact of this discovery on basic and clinical physiology is becoming obvious. Since publication of the first molecular water channel (Preston *et al.,* 1992), more than 700 reports have appeared in the scientific and clinical literature, and it is clear that aquaporin research will advance our understanding of diverse physiological and pathophysiological processes in humans, other vertebrates, invertebrates, microbials, and plants. It is humbling to see that a single, unexpected observation can still provide new insights that alter the course of biological research. Nevertheless, the greater importance of our work is often not obvious, and it is worth remembering a gentle caution issued a century ago:

> In summary, there are no small problems. Problems that appear small are large problems that are not understood. Nature is a harmonious mechanism where all parts, including those appearing to play a secondary role, cooperate in the functional whole. No one can predict their importance in the future. All natural arrangements, however capricious they may seem, have a function.
>
> —Santiago Ramón y Cajal, 1897, *Advice for a Young Investigator*

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