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Update on Cell Biology

Aquaporins: The Molecular Basis of Facilitated Water Movement Through Living Plant Cells?[1]

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Osmotic effects observed with living cells indicate that their plasma membranes are freely permeable to water while essentially creating a barrier to other molecules. The hydraulic conductivity of biological membranes is sometimes still ascribed to the simple diffusion of water molecules through the lipid bilayer. However, more than 30 years ago the idea was advanced that hydraulic water movement through living cells occurs by bulk flow of water through pores in the membrane (Sidel and Solomon, 1957). Certain membranes of animal cells are unusually permeable to water and there is now a substantial body of evidence for the existence of water transport channels in such membranes (reviewed by Finkelstein, 1987; Verkman, 1992). For example, membranes from red blood cells and renal proximal tubules are exceptionally permeable to water; the membranes of the convoluted distal tubules of the kidney are also highly water permeable, and water permeability can be modulated by hormones. The hydraulic conductivity of plant cells has been thoroughly investigated and numerous reviews on this subject have been published (for a recent review, see Steudle, 1992).

Many observations on plants and animals support the recent discovery of proteins that form water channels (Preston et al., 1992; Fushimi et al., 1993; Maurel et al., 1993). We proposed to call such proteins "aquaporins" (Agre et al., 1993). These water channel proteins belong to the MIP family, an ancient family of membrane proteins (see Reizer et al., 1993, for review). Aquaporins form water-selective channels, allowing water to pass freely while excluding ions and metabolites. In plants, aquaporins have been demonstrated in the tonoplast (vacuolar membrane) (Höfte et al., 1992), but they also may be present in the plasma membrane (Kammerloher and Schäffner, 1993). In animal cells, aquaporins are found in the plasma membranes of specific cell types. It is important to note that such channels permit or facilitate the movement of water through membranes and do not act as pumps. The driving forces behind water movement are hydraulic or osmotic in nature.

AQUAPORINS ARE MEMBERS OF AN ANCIENT FAMILY OF CHANNEL PROTEINS

In earlier reports, we and others have described in plants a family of integral membrane proteins that has cognates in mammals, yeasts, and bacteria and is part of the larger MIP family (see Reizer et al., 1993, for a recent review). The polypeptide chains of all the MIPs span the membrane six times and have amino and carboxy termini that face the cytoplasm (Fig. 1). In plants, this family is represented by several TIPs, such as α-TIP and γ-TIP, the soybean nodule protein NOD26, proteins such as Rtob7 that are specifically expressed in roots, and proteins that are induced by specific stresses such as irradiation or dehydration or by darkness.

The functions of many MIPs are still unknown, although most of the proteins have been postulated to be involved in transport processes. Several MIPs, such as plant γ-TIP and CHIP28, have been shown to be aquaporins, whereas GlpF and MIP itself transport small polyols and ions, respectively. Other members of the MIP family have properties or cellular locations that are suggestive of a transport function: NOD26 is in the peribacteroid membrane of soybean nodules and may allow an exchange of metabolites between the bacteroids and the cytoplasm; α-TIP is found in the protein body membranes of seeds and may play a role in transport processes into or out of the protein bodies during seed development or germination. BIB, the big brain protein of *Drosophila melanogaster*, is required for normal brain development and may play a role in cell-to-cell communication.

Phylogenetic trees depict the relatedness of the various MIP family proteins as well as their clustering patterns. On such a tree the eight plant proteins and the five animal (mammalian and *Drosophila*) proteins form respectively two clusters, whereas the yeast and bacterial proteins appear less related (Reizer et al., 1993). A phylogenetic tree of the plant proteins shows that proteins with similar patterns of expression, such as the seed-specific α-TIPs or the desiccation-induced proteins, cluster together (Höfte et al., 1992). Eukaryotic species contain several MIP genes—eight full-length TIP sequences have already been obtained from *Arabidopsis thaliana*, with more probably to come—whereas only a single MIP gene (*GLP*) has ever been found in bacteria. This led Reizer et al. (1993) to speculate that a single MIP gene was

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Abbreviations: MIP, major intrinsic protein; TIP, tonoplast intrinsic protein.

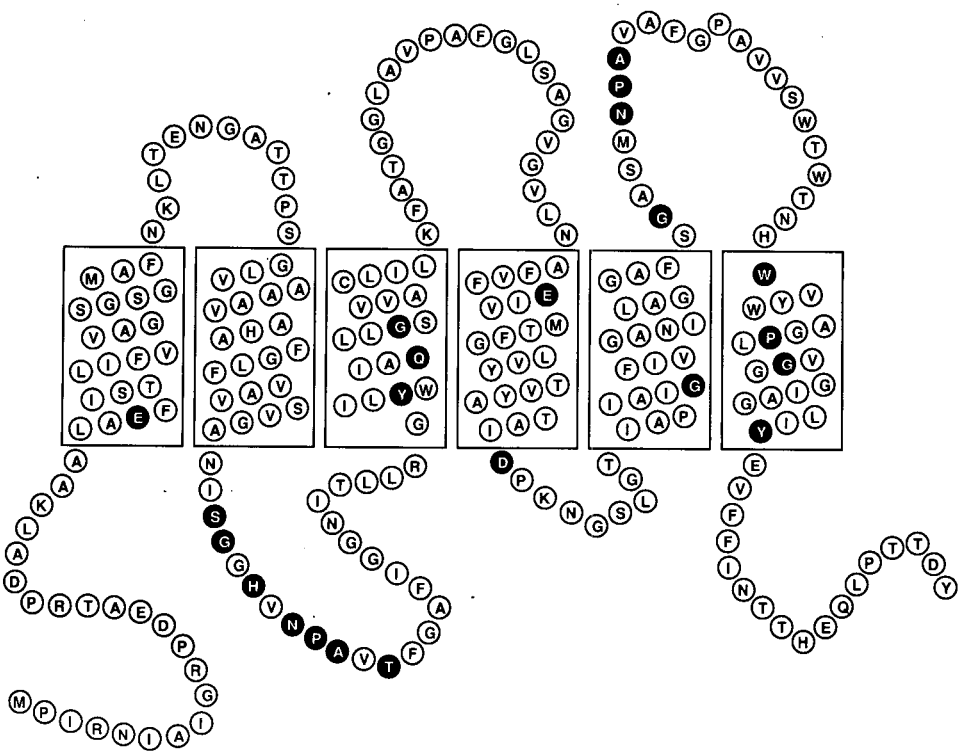

Figure 1. Schematic representation of aquaporin γ-TIP in the tonoplast showing the six putative transmembrane domains. The orientation of the protein is based primarily on extensive studies with the bovine lens protein MIP; the orientation of the sixth transmembrane domain has been confirmed for the seed protein α-TIP. The residues that are conserved in all members of the MIP family are shown as dark circles.

vertically transmitted from the prokaryotes to the different eukaryotic kingdoms, and that these genes then duplicated and diverged to yield the different subfamilies.

THE EXISTENCE OF WATER CHANNEL PROTEINS HAS BEEN KNOWN FOR MANY YEARS

Bulk flow of water across a membrane occurs in response to an osmotic or hydrostatic gradient. Osmotic water permeability is readily measured in small vesicles or cells by the stopped-flow light-scattering technique, a method that relies on the dependence of light scattering on vesicle or cell volume, and is used to quantitate the time course of net water flow that occurs in response to transmembrane osmotic gradients. The osmotic gradients are established by adding an impermeant solute to the external solution. With the help of other chemical and physical methods (tracer fluxes, ^{1}H-NMR) to measure diffusional and osmotic water transport across biological membranes, biophysicists and cell physiologists have obtained evidence for the existence of facilitated or channel-mediated water transport in several membranes (see Macey, 1984; Finkelstein, 1987). Membranes with facilitated water transport share a number of properties (Macey, 1984; Verkman, 1992) that generally support but do not prove the presence of water channels. For example, water transport across these membranes is inhibited by mercurial sulfhydryl reagents, demonstrating the existence of proteinaceous components in water channels, and the functional unit of the water channel in kidney tubules and red blood cells is 30 kD, as determined by radiation inactivation (van Hoek et al., 1991).

In spite of this accumulated knowledge about the channels that facilitate water movement, until recently no one succeeded in identifying or isolating any candidate proteins. Expression of mRNAs in *Xenopus* oocytes led to the identification of water channel proteins in red blood cells (Preston et al., 1992), kidney tubules (Fushimi et al., 1993; Zhang et al., 1993a), and plants (Maurel et al., 1993).

THE ACTIVITY OF AQUAPORINS CAN BE READILY DEMONSTRATED BY SWELLING ASSAYS WITH *XENOPUS* OOCYTES

Xenopus oocytes are very large cells, measuring 600 to 1300 μm in diameter, that can be micromanipulated with relative ease. This and their high RNA translation capacity make it possible to inject oocytes with exogenous mRNA to study the expression of heterologous proteins. Because the plasma membrane of the oocyte appears to have a low water permeability, these cells are suitable to study the activity of water channel proteins. The common procedure to assay for aquaporin activity is to shift oocytes 2 to 3 d after injecting them with aquaporin mRNA to a hypotonic culture medium by diluting the medium 3-fold and to measure the influx of water by determining the changes in cell volume after the shift. The presence of aquaporin in the plasma membrane

increases the osmotic water permeability, Pf, from 0.1×10^{-2} cm/s to 1.0 to 2.0×10^{-2} cm/s. This causes the oocytes to increase in volume by 50% and to rupture within 2 to 6 min of hypotonic exposure, whereas control oocytes burst after 45 to 60 min. It is the large size of the oocytes that makes these observations easy. The diameter of an oocyte is 50 times larger than that of a vacuole and 200 times larger than that of a red blood cell. Since the surface-to-volume ratio decreases with increased cell size, and assuming similar rates of water uptake per unit area of membrane, then a vacuole would burst in 5 to 6 s and a red cell in about 1 s under conditions where it would take oocytes several minutes to swell and burst.

The mRNAs that have been identified with the oocyte swelling assay as encoding high-activity water channels all encode proteins that are members of the MIP family. Significantly, mercurial sulfhydryl reagents inhibit the increase in Pf caused by the presence of the aquaporins, just as they inhibit the permeability of red cell and kidney cell membranes. In addition, all aquaporins have a molecular mass of approximately 27 kD, which is close to the size of the functional unit of the water channel as determined by radiation inactivation. Together, these observations strongly support the conclusion that the aquaporins are the proteins that permit the rapid flow of water through biological membranes.

AQUAPORINS FORM WATER-SELECTIVE CHANNELS

Swelling of oocytes in the manner described above has been obtained with mRNAs encoding the animal proteins CHIP28 (Preston et al., 1992; Zhang et al., 1993a) and WChP (Fushimi et al., 1993), as well as two plant proteins, γ-TIP (Maurel et al., 1993) and RD28 (Chrispeels et al., 1994). The water transport property of CHIP28 has been confirmed with liposomes in which purified CHIP28 had been incorporated, but such reconstitution experiments have not yet been done for any of the plant proteins.

Generally, efforts to demonstrate the existence of transport activities associated with aquaporins other than water transport have been fruitless. In some of these experiments, oocytes that express aquaporins were used for voltage clamp studies or to measure uptake of radioactive metabolites. No ions or metabolites have been found to be co-transported with water (Maurel et al., 1994). Conversely, homologs such as GlpF that transport glycerol do not transport water. Expression of GlpF in oocytes allows them to take up glycerol in accordance with the known function of GlpF but does not induce swelling when the oocytes are shifted to a hypoosmotic medium (Maurel et al., 1994). It appears, therefore, that aquaporins are channels highly selective for water. However, other channel proteins have been shown to transport water and ions together. Electro-osmotic couplings of up to 200 mol of water per mol of cation have been reported for *Nitella* (see Lüttge and Higinbotham, 1979). Red beet vacuoles have a stretch-activated channel that is sensitive to osmotic pressure and may mediate cation- and osmotically driven water fluxes (Alexandre and Lassalles, 1992).

WHAT IS THE MOLECULAR MECHANISM OF WATER TRANSPORT?

Amino acid sequence comparisons of all members of the MIP family show that there are 22 amino acids that are conserved in all sequences; these are shown in dark circles in Figure 1. These amino acids include a membrane-embedded glutamate (E) in transmembrane segments 1 and 4, a sequence of Asn-Pro-Ala (NPA) in the loops between transmembrane segments 2 and 3 as well as between transmembrane segments 5 and 6 (these loops are on opposite sides of the membrane), and a Gly (G) in the middle of transmembrane segments 3 and 6 (Reizer et al., 1993; Fig. 1). A detailed computer analysis of the first and second halves of many MIPs shows that the full sequence probably arose by an intragenic duplication event (Reizer et al., 1993). Most interesting is that the two similar halves have opposite orientations in the membrane: NPA is inside in the first half of the molecule but outside in the second half. This generates a symmetry of the channel with respect to both sides of the membrane.

The low activation energy for water transport through membranes that contain aquaporins (E_a = 4–6 kcal/mol) is similar to the activation energy for the self diffusion of water or for the viscous transport of water. This implies that as water molecules traverse the water channel, they encounter polar groups similar to the polar environment of the bulk solution external to the membrane (Macey, 1984). Macey (1984) postulated that the water channel should have a radial dimension that lies between 1.5 Å (the radius of a water molecule) and 2.0 Å (the radius of a urea molecule) and that water is constrained to move as a single file of molecules. The ratio of the osmotic to the diffusional permeability indicates that five to nine water molecules should be in the rate-limiting portion of the channel (Finkelstein, 1987).

Water channels are characteristically inhibited by mercury derivatives, and this inhibition is reversed by reducing reagents (Macey, 1984). This is also the case for the swelling of oocytes that express plant or animal aquaporins (γ-TIP or CHIP28). Such a result suggests the functional importance of Cys or Met residue(s). CHIP has several Cys or Met residues, and the mercury-sensitive amino acid residue of CHIP28 has been identified recently as Cys^{189} (Preston et al., 1993; Zhang et al., 1993b). This residue is located adjacent to the conserved NPA motif in the loop between membrane-spanning domains 5 and 6. Substitution of Cys^{189} in CHIP28 by a Ser residue creates an active water channel that cannot be inhibited by mercury (Preston et al., 1993). Replacement of Cys^{189} by large amino acids such as Val or Trp abrogates water transport. Of the four aquaporins that have been tested (CHIP28, WChP, γ-TIP, and RD28), only RD28 is insensitive to mercury, and it has no Cys or Met residue close to the second conserved NPA motif (Yamaguchi-Shinozaki et al., 1992).

Both MIP26 and CHIP28 (Smith and Agre, 1991) form tetramers, but the monomer appears to be the functional unit of water transport. This is supported by radiation inactivation data (van Hoek et al., 1991) and by the co-expression of CHIP28 and a nonfunctional mutant, such as the $Cys^{189} \rightarrow Tyr^{189}$ mutation. Mutant inactive protein is not negatively

dominant and the rate of water transport is proportional to the amount of active protein (Preston et al., 1993; Zhang et al., 1993b).

EXPRESSION AND SUBCELLULAR LOCATION OF PLANT AQUAPORINS

The expression pattern of a gene and the subcellular location of the protein it encodes can often give additional clues about the physiological role of the protein. The aquaporin γ-TIP is located in the tonoplast and the gene is most highly expressed either during or immediately after cell elongation (Ludevid et al., 1992). Expression is also higher in the vascular bundles than in the leaf parenchyma or cortex (Ludevid et al., 1992). There is no expression in the root and stem meristems, although these cells have numerous small vacuoles. A detailed study is needed to describe exactly when γ-TIP accumulates: during the vacuolation process or immediately thereafter. The cells of growing tissues require a considerable supply of water and have a high hydraulic conductivity (Cosgrove and Steudle, 1981; Steudle and Boyer, 1985), and this finding is in agreement with the high expression of γ-TIP in such cells.

The aquaporin RD28 identified by Yamaguchi-Shinozaki et al. (1992) is induced by desiccation, and preliminary evidence indicates that it may be a plasma membrane protein, rather than a tonoplast protein (Kammerloher and Schäffner, 1993). Thus, it may be involved in permitting enhanced water flow under conditions of water stress. A homolog of this protein has been found in pea shoots (clone 7A, Guerrero et al., 1990), and two other homologs are present in *A. thaliana*.

DO AQUAPORINS PLAY A ROLE IN WATER MOVEMENT WITHIN THE PLANT?

In most plants, a transpiration stream that starts with the uptake of water from the soil and ends with the loss of water vapor from the leaves moves through the plant during daylight hours. Movement through the xylem vessels obviously does not involve aquaporins, since the vessel elements have no membranes, but how does water get into the xylem and how does it flow from the xylem to and through other cells? There are two possible pathways for osmotic flow of water between tissues: an apoplastic route that encompasses only the cell walls and a symplastic and transcellular path that involves the cytoplasm as well as the vacuoles (Fig. 2). This distinction is somewhat artificial because it is likely that water moves via both paths at all times. Measurements obtained with pressure probes indicate that the preferred route may depend on the species, the plant organ, the physiological condition of the plant, as well as the driving force (hydrostatic or osmotic pressure). In maize and cotton roots, apoplastic transport dominates, but in barley and bean, transport is mostly cell to cell (see Steudle, 1992, for review and refs. therein). Plasma membranes are thought to be the primary impediments to water flow, with the role of plasmodesmata still poorly understood. The presence of aquaporins in the tonoplast increases the effective cellular cross-section through which water flows freely once it has passed through the plasma membrane.

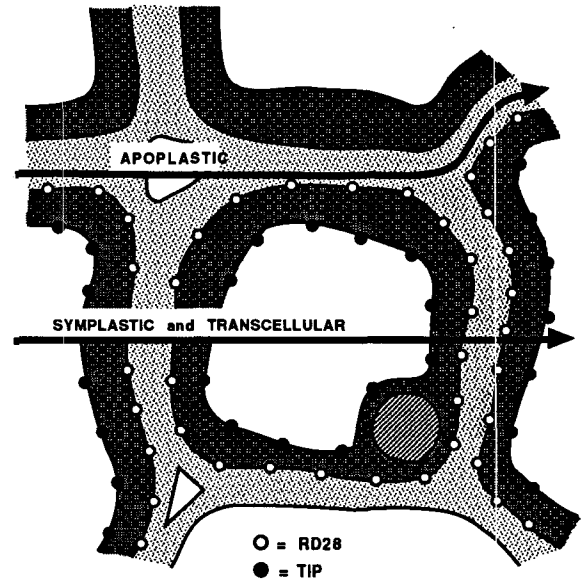

Figure 2. Possible routes for hydraulic water flow through a living tissue: apoplastic and symplastic/transcellular. TIP, shown in the tonoplast, is constitutively expressed, whereas RD28, shown in the plasma membrane, is expressed only as a result of desiccation.

Water flow through the cells could be regulated in two different ways: first, by altering the activity of the individual water channel proteins, and second, by changing the abundance of these proteins in the membranes. Down-regulating tonoplast aquaporins should decrease the cell-to-cell hydraulic conductivity. To increase overall transcellular water conductivity, a cell could also synthesize new aquaporins and target these to the plasma membrane. This may happen during water stress when the synthesis of the plasma membrane-localized RD28 is induced. When organs dry out, the presence of aquaporins in the plasma membrane may well facilitate supplying the living cells with water from the xylem. Steudle and Boyer (1985) suggested that the rate of water movement out of the xylem may be determined by the small cells that surround the vessel elements. Thus, the control of water flow may operate only in those cells, and the expression of γ-TIP as well as the induction of RD28 may be higher in the vascular bundles than in other cells. Results obtained with promoter-β-glucuronidase fusions with the γ-TIP promoter (Ludevid et al., 1992) are consistent with this interpretation. The presence of aquaporins in the cortex and mesophyll could also significantly reduce the resistance to water flow through the plant.

Water channels may be involved in other processes related to water absorption and flow within the plant tissues. Processes such as pollen or seed imbibition and germination and stomatal opening and closing will have to be revisited in the light of recent evidence for water channels in plants. The cells of growing tissues require a considerable supply of water and have a high hydraulic conductivity. Expression of aquaporins may be highest in these cells. The use of the existing aquaporin probes, coupled with the oocyte swelling assay, will aid the discovery of new plant aquaporins.

WHERE DO WE GO FROM HERE?

The identification of aquaporins has opened an exciting new chapter in plant-water relations. We can envision how water flow within the plant could be modified by altering the regulation, expression, or subcellular location of specific aquaporins. We now need to establish that aquaporins are the molecules that permit and modulate cell-to-cell water flow and the hydraulic conductivity of the plant. This can be done with transgenic plants that express different forms of these proteins (e.g. mercury sensitive and mercury insensitive) in different membranes. By combining molecular biology with plant physiology, it should be possible to determine the role that aquaporins play in water transport in the plant. It will be of major interest to determine what is the limiting factor: the generation of water potential gradients or the hydraulic conductivity of the cellular membranes.

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