Functional Expression of AQP3 in Human Skin Epidermis and Reconstructed Epidermis

Rachid Sougrat, Maryse Morand,* Catherine Gondran,* Patrick Barré,* R. Gobin, Frediric Bonté,* Mara Dumas,* and Jean-Marc Verbavatz

Service de Biologie Cellulaire, CEA/Saclay, Gif-sur-Yvette, France; *LVMH Branche Parfums et Cosmétiques, Laboratoire R&D, Saint Jean de Braye, France

The purpose of this study was to examine the presence of aquaporin water channels in human skin and to assess their functional role. On western blots of human epidermis obtained from plastic surgery, a strong signal was obtained with polyclonal anti-aquaporin-3 antibodies. By indirect immunofluorescence on 5 μm cryosections, anti-aquaporin-3 antibodies strongly stained keratinocyte plasma membranes in human epidermis, whereas no staining was observed in the dermis or the stratum corneum or when anti-aquaporin-3 antibodies were preabsorbed with the peptide used for immunization. Similarly, a strong signal with anti-aquaporin-3 antibodies was observed in keratinocyte plasma membranes of reconstructed human epidermis in culture at the air–liquid interface for up to 3 wk. The keratinocyte plasma membrane localization of aquaporin-3 was confirmed at the electron microscope level in prickle cells. In addition an intracellular localization of aquaporin-3 was also detected in epidermis basal cells. Osmotically induced transepidermal water permeability was measured on stripped human skin and on reconstructed epidermis. Water transport across both stripped human skin and 2–3 wk reconstructed epidermis was comparable, inhibited by > 50% by 1 mM $HgCl_2$ and fully inhibited by acid pH. By stopped-flow light scattering, keratinocyte plasma membranes, where aquaporin-3 is localized, exhibited a high, pH-sensitive, water permeability. Although human skin is highly impermeable to water, this is primarily accounted for by the stratum corneum, where a steep water content gradient was demonstrated. In contrast, the water content of viable strata of the epidermis is remarkably constant. Our results suggest that the human epidermis, below the stratum corneum, exhibits a high, aquaporin-3-mediated, water permeability. We propose that the role of aquaporin-3 is to water-clamp viable layers of the epidermis in order to improve the hydration of the epidermis below the stratum corneum. ***Key words: aquaporin water channels/human keratinocytes/water permeability. J Invest Dermatol 118:678–685, 2002***

Aquaporins are a family of molecular water channels, 10 of which have been cloned in mammals and many more have been found in lower organisms (yeast, bacteria), plants, insects, and amphibians (Borgnia *et al*, 1999; Verkman and Mitra, 2000). The most studied water channel, aquaporin-1 (AQP1), is a 28 kDa glycoprotein, cloned from human red blood cells (Preston *et al*, 1992), that forms tetramers within membranes (Verbavatz *et al*, 1993) in which the monomers are independently functional (Shi *et al*, 1994). Each AQP1 monomer consists of six hydrophobic transmembrane domains, believed to surround a narrow pore specific for water (Murata *et al*, 2000; Ren *et al*, 2000). AQP1 is expressed in many cell types including red blood cells, kidney tubules, choroid plexus, and other epithelia and endothelia (Sabolic *et al*, 1992; Nielsen *et al*, 1993a; 1993b), where it is primarily and constitutively localized at plasma membranes. Whereas some aquaporins, like aquaporin-2 in kidney-collecting duct or aquaporin-0 (or MIP) in lens fibers, have been detected only in specific tissues, other aquaporins also have a relatively wide distribution in mammals. In rat, aquaporin-3 (AQP3) was detected in kidney, brain, trachea, colon, urinary bladder, skin epidermis (Frigeri *et al*, 1995; Matsuzaki *et al*, 1999), and red blood cells (Roudier *et al*, 1998).

The functional role of aquaporins has been most studied in red blood cells (Mathai *et al*, 1996; Roudier *et al*, 1998) and kidney (Deen *et al*, 1994; Chou *et al*, 1998; Ma *et al*, 1998; 2000). In addition to water permeability, some aquaporins, including AQP3, are also permeable to small solutes such as glycerol and urea (Roudier *et al*, 1998; Zeuthen and Klaerke, 1999; Ma *et al*, 2000) but the relevance of these additional properties has not yet been demonstrated. The physiologic importance of aquaporins, particularly in epithelial cells involved in water and solute transport, has been demonstrated (van Os *et al*, 2000; Verkman *et al*, 2000). In humans, mutations in AQP2, resulting in low kidney-collecting-duct water permeability and hypotonic urine excretion, are responsible for hereditary nephrogenic diabetes insipidus (Deen *et al*, 1994). Transgenic mice lacking AQP4 expression showed a defect in urinary concentration (Chou *et al*, 1998). Although no phenotype was detected in humans lacking functional AQP1 initially (Mathai *et al*, 1996), studies in AQP1 null mice demonstrate an important role for AQP1 in kidney (Ma *et al*, 1998) and lung (Bai *et al*, 1999). Recently, a defect in urinary concentration in humans lacking AQP1 was confirmed (King *et al*, 2001). Finally,

Manuscript received August 7, 2001; revised October 22, 2001; accepted for publication November 16, 2001.

Reprint requests to: Dr. Jean-Marc Verbavatz, DBCM/SBCe, Bât. 532, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France. Email: jmverbavatz @cea.fr

mice lacking AQP3 were markedly polyuric due to defective water reabsorption in kidney (Ma *et al*, 2000). Yet, in some tissues, like skeletal muscle, the physiologic role of aquaporins has been controversial (Frigeri *et al*, 1998; Yang *et al*, 2000).

In other tissues, including skin epidermis (Frigeri *et al*, 1995; Matsuzaki *et al*, 1999), the expression of aquaporins has only been demonstrated in rat and their exact localization and/or functional role remains uncertain. The skin, in particular, is a functional and physical barrier against water exchange between the body and the environment. Therefore, unlike epithelia involved in water and solute transport or exocrine glands, the expression of aquaporins, which are believed to facilitate water transport along a favorable osmotic gradient, may appear as a surprising finding in skin epidermis. Indeed, because the water content drops sharply across the epidermis, water channels in this tissue could result in a massive transepidermal water loss. The epidermis is a pluristratified epithelium, primarily composed of keratinocytes. Keratinocytes undergo continuous proliferation and terminally differentiate into cornified cells that constitute the stratum corneum (SC). It is established that the SC is the primary barrier to transepidermal water transport in the epidermis (Landmann, 1988; Warner *et al*, 1988), but little is known on fluid transport within viable layers of the epidermis.

The purpose of this work was to examine the expression and localization of aquaporin water channels in human skin and to determine whether molecular water channels may play a significant functional role in this tissue. The expression and function of aquaporins in epidermis reconstructed from human keratinocytes grown in culture were also investigated. We conclude that, in viable layers of the human epidermis, AQP3 is abundant in keratinocyte plasma membranes. Keratinocyte membranes exhibited a high water permeability, confirming that AQP3 is functional. In addition, despite the thickness of this tissue a significant, pH-sensitive, osmotic water permeability was detected across viable layers of the epidermis. This water permeability was consistent with a role for AQP3 in this tissue. Finally, abundant expression of AQP3 and a similar osmotic permeability were also observed in reconstructed epidermis, suggesting that reconstructed epidermis may be a useful *in vitro* model of human epidermis.

MATERIALS AND METHODS

Tissues and reconstructed epidermis Normal human skin biopsies were obtained from donors after facial or abdominal plastic surgery. Tissue samples were conserved in phosphate-buffered saline (PBS) containing 2% fetal bovine serum, 100 μg per ml penicillin, and 100 μg per ml streptomycin until they reached the laboratory (within 24 h). For cell culture, keratinocytes were isolated by treatment of skin samples with 0.25% trypsin for 1 h, followed by centrifugation in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The pellet of human keratinocytes was resuspended in keratinocyte-SFM (Gibco) culture medium and cells were grown on permeable filters (Transwell 3 μm Costar) for 3–4 d until confluence. Then (day 0), cells were grown at the air–liquid interface in DMEM:HAM-F_{12} (3:1) and allowed to differentiate into multilayered, reconstructed epidermis (Noel-Hudson *et al*, 1995).

Reproducible removal of the SC was achieved by 20 successive tape-strippings using pressure-sensitive adhesive-coated disks (D-Squame, Monaderm, Monaco), applied for 5 s.

Antibodies Anti-AQP1 antibodies were polyclonal antibodies raised in rabbits against purified human AQP1. Anti-AQP2, anti-AQP3, anti-AQP4, and anti-AQP5 antibodies were affinity-purified polyclonal antibodies raised in rabbits against the C-ter peptide sequence of the rat aquaporin isoform, which cross-react with the equivalent human protein. The anti-AQP3 antibody was previously characterized by Roudier *et al* (1998).

Western blotting For western blotting, reconstructed epidermis, obtained from human keratinocyte cultures or human skin fragments (from plastic surgery), were homogenized in PBS containing 1 mM phenylmethylsulfonyl fluoride. The protein concentration of each sample was determined with a commercially available kit (Micro BCA, Pierce), diluted to a final concentration of 1 mg per ml, and solubilized in Laemmli sample buffer. 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with constant amounts (10 μg per lane) of samples and proteins were transferred to PVDF membranes. Western blots were preincubated in PBS containing 5% nonfat dry milk (PBS/milk), incubated for 1 h in primary polyclonal antibodies diluted in PBS/milk, in the absence or presence of 100 μg per ml of the synthetic peptide used for immunization, and washed 3 × 15 min in PBS/milk. Membranes were then incubated in a 1:5000 dilution of peroxidase-conjugated antirabbit polyclonal antibodies in PBS/milk, washed 2 × 15 min and 1 × 1 h in PBS/milk, and then washed 2 × 5 min in PBS containing 0.02% Tween-20. Antibody staining was revealed by enhanced chemiluminescence.

Indirect immunofluorescence For indirect immunofluorescence, human skin fragments or commercially available reconstructed human epidermis grown in culture in a modified chemically defined medium MCDBD 153 on inert microporous polycarbonate filters at the air–liquid interface for 16 d (Skinethic® Laboratory, Nice, France), were fixed overnight by immersion in PBS containing 4% paraformaldehyde and washed extensively in PBS. Tissues were infiltrated overnight in 30% sucrose and frozen in liquid nitrogen, and 5 μm cryostat sections were collected on SuperFrost Plus glass slides. Sections were preincubated in PBS containing 1% bovine serum albumin (PBS/BSA) for 5 min, then in rabbit polyclonal antibodies against AQP3 (antisera diluted 1:500, with or without 100 μg per ml immunizing peptide) in PBS/BSA for 1 h, and washed 3 × 10 min in PBS/BSA. Slides were then incubated for 45 min in a 1:100 dilution of fluorescein-isothiocyanate-conjugated goat-antirabbit antibodies (AQP3), washed 3 × 10 min, counterstained with Evans blue, and mounted in 50% glycerol containing 2% n-propyl gallate before observation in the fluorescence microscope (van OX AH-2, Olympus).

For double staining for AQP3 and keratin 14, the staining for AQP3 was followed by a 1 h incubation in 1 μg per ml monoclonal anti-keratin 14 (in PBS/BSA), 3 × 5 min washes, and a 45 min incubation in Cy3-coupled goat-antimouse antibodies; no counterstaining was performed.

Electron microscopy gold labeling For the localization of AQP3 at the electron microscope level, human skin samples were fixed in 4% paraformaldehyde in PBS containing 0.1% glutaraldehyde and washed several times in PBS. Small pieces of skin were embedded in Unicryl and 90 nm thick sections were cut with the ultracryomicrotome (UltraCut S, Leica) and collected on nickel formvar-coated electron microscope grids. The sections were preincubated for 20 min in 20 mM Tris buffer (pH 7.4) containing 0.1% BSA, 0.1% fish gelatin, 0.05% Tween-20, and then incubated in affinity-purified polyclonal anti-AQP3 antibodies (1:50 dilution) in Tris buffer for 1 h. The sections were washed, and incubated in a 1:25 dilution of 10 nm colloidal gold-conjugated goat-antirabbit antibodies (Amersham) in Tris buffer for 1 h and washed 6 × 5 min. Sections were stained in uranyl acetate, coated with methylcellulose, and observed in the electron microscope (EM 400, Philips).

Water permeability The osmotic water permeability of human skin and reconstructed epidermis in culture was studied with a previously described apparatus (Dorr *et al*, 1997), designed for transepithelial water flow measurements across epithelial cells in isolated tissues (Bourguet and Jard, 1964) or confluent cells in culture grown on permeable filters (Toriano *et al*, 1998). Because no water flux was detectable across intact human skin, measurements were performed on stripped human epidermis in order to investigate fluid movements in viable layers of the epidermis. Briefly, the tissue (isolated stripped skin epidermis or reconstructed epidermis grown on a permeable filter) was mounted in a Ussing chamber at 37°C. The basal side of the preparation was bathed in culture medium (DMEM:HAM-F_{12} 3:1), without serum and the apical side of the preparation, initially bathed in the same medium, was then exposed to a 2-fold dilution of this solution with water, thus creating a 150 mOsm osmotic gradient; the osmotically induced water fluxes were recorded every minute, with a resolution of ≈40 nl.

Keratinocyte membrane vesicle preparation Epidermis isolated from normal human skin biopsies was suspended in PBS containing a protease inhibitor cocktail (P8340, Sigma) to make a 20% (wt/vol) homogenate. The isolated epidermis was homogenized by Polytron (4 × 30 s), submitted to two freeze–thaw cycles, homogenized with a Dounce (A pestle), followed by sonication, and finally homogenized with a tight B pestle. The homogenate was centrifuged twice for 20 min at 1500 × *g*. The supernatant was then centrifuged for 1 h at 80,000 × *g*. The pellet was resuspended in PBS containing 10 mM ethylenediamine tetraacetic acid and filtered at 0.22 μm.

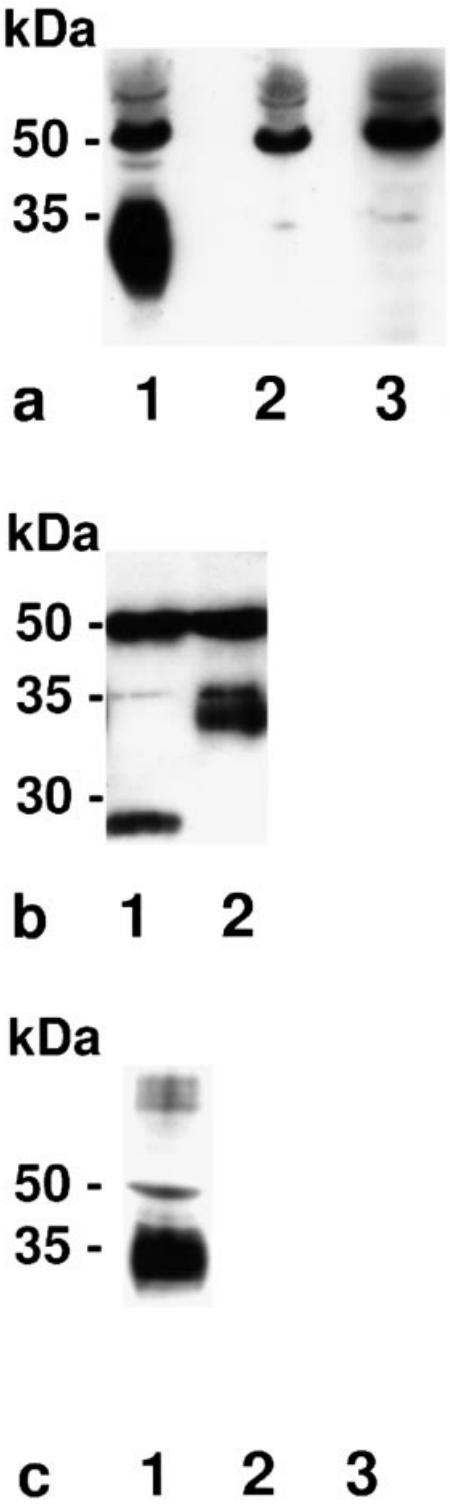

Figure 1. Immunoblots of AQP3. (*a*) In human epidermis homogenates, affinity-purified anti-AQP3 antibodies specifically recognized a diffuse, glycosylated band between 30 and 35 kDa (*lane 1*). This band was absent when the antibodies were preabsorbed with the immune peptide (*lane 2*) or in the absence of primary antibodies (*lane 3*). (*b*) The broad band detected in human skin epidermis by anti-AQP3 antiserum (*lane 2*) was shifted to ≈27 kDa after deglycosylation with PNGase-F (*lane 1*). (*c*) In reconstructed human epidermis the broad glycosylated band of 30–35 kDa was also revealed by affinity-purified anti-AQP3 antibodies (*lane 1*). Unlike in native human epidermis, a weak but specific 50 kDa band was also detected as shown by the absence of staining when anti-AQP3 antibodies were preabsorbed with the peptide (*lane 2*) or absent (*lane 3*). The 50 kDa band may represent AQP3 dimers.

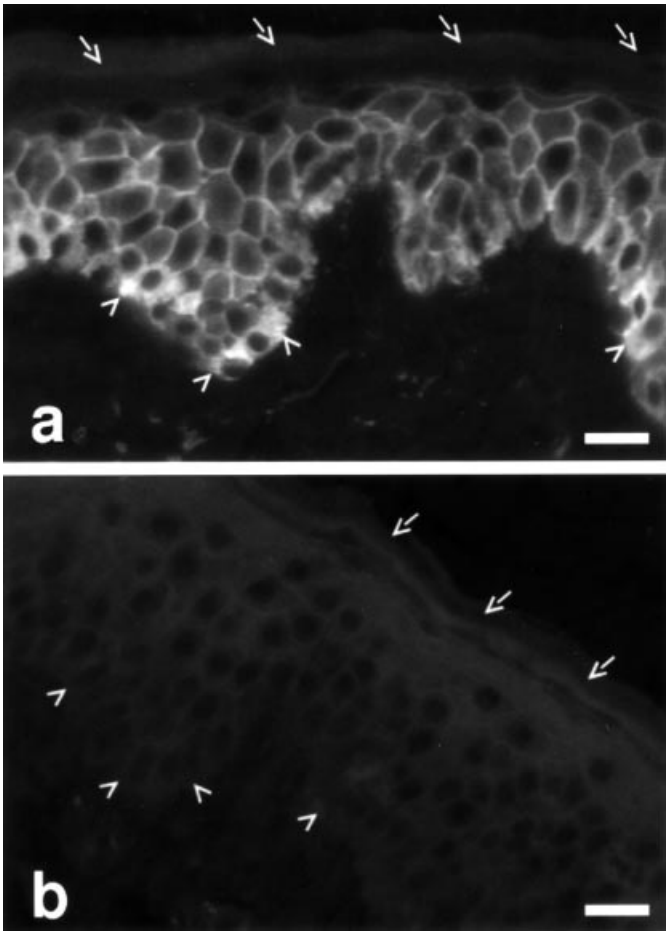

Figure 2. Immunofluorescence localization of AQP3 in human skin. On 5 μm human skin cryosections, polyclonal anti-AQP3 antibodies strongly stained keratinocyte plasma membranes (*a*). The SC (*arrows*) and the dermis were unstained. Some intracellular staining was observed in basal cells of the epidermis (*arrowheads*). No staining was observed when antibodies were incubated in the presence of the AQP3 C-ter peptide used for immunization (*b*). *Scale bar*: 20 μm.

Stopped-flow light scattering Experiments were carried out on keratinocyte membrane vesicles at 10°C, in order to minimize lipidic water permeability. The time course of vesicle shrinkage following a 250 mOsm sucrose osmotic shock was recorded by 90° light scattering (at 534 nm) in a stopped-flow spectrophotometer (SFM3, Biologic, Claix, France). Data from three consecutive shoots were averaged and fitted to single exponential functions by BIOKINE software (Biologic, France). The osmotic water permeability coefficient (P_f in cm per s) was determined from the following equation as previously reported (Roudier *et al*, 1998): $dV(t)/dt = P_f(S/V_0)\mathrm{Mw}[C_i/V(t)C_o]$ where $V(t)$ is the relative volume of vesicles as a function of time, S/V_0 is the initial vesicle surface area to volume ratio, determined from freeze-fracture electron microscope observations (average diameter 90 ± 20 nm, n = 47), Mw is the molar volume of water, and C_i, C_o are the initial intravesicular and extravesicular concentrations of solute.

RESULTS

Western blotting In human skin homogenates (**Fig 1*a***), anti-AQP3 polyclonal antipeptide antibodies (1:5000 dilution of whole rabbit antiserum, or affinity-purified antibodies) strongly and specifically stained a diffuse band between 30 and 35 kDa (*lane 1*). An additional specie of ≈50 kDa band was also detected when the serum was preabsorbed with the peptide used for immunization or in the absence of primary antibody (**Fig 1*a***, *lanes 2*, *3*, respectively). In deglycosylation experiments (**Fig 1*b***), following 18 h treatment with PNGase-F, the broad band of 30–35 kDa (**Fig 1*b***, *lane 2*) was

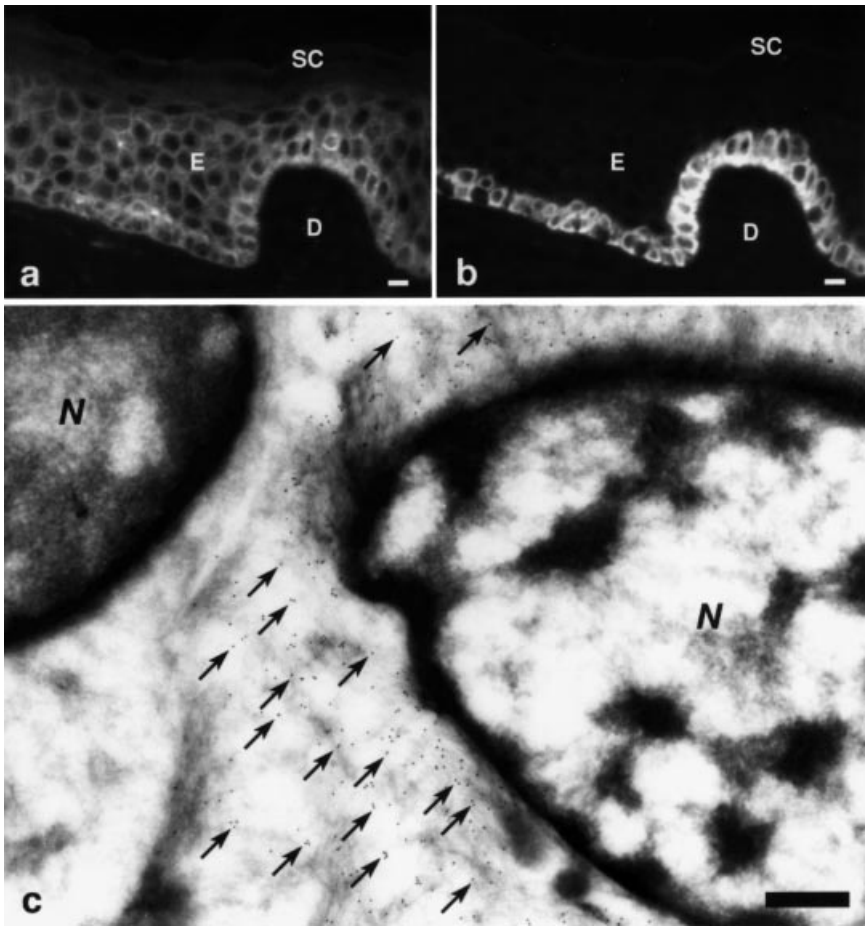

Figure 3. AQP3/keratin 14 double staining. Human skin cryosections were double-stained for AQP3 and keratin 14. Anti-AQP3 antibodies stained the epidermis with a strong signal in the basal layer (*a*). Anti-keratin-14 antibodies strongly stained the basal layer of the epidermis for which it is specific (*b*). On Unicryl thin sections, electron microscope gold labeling of AQP3 in basal cells of the epidermis shows intracellular localization (*c*, *arrows*). D, dermis; E, epidermis SC. *Scale bar*: (*a*, *b*) 10 μm; (*c*) 350 nm.

identified as a glycosylated form of the human AQP3 monomer of ≈26 kDa (**Fig 1*b***, *lane 1*). This pattern of staining is consistent with what was previously described for human AQP3 (Roudier *et al*, 1998). AQP1, AQP2, AQP4, and AQP5 were not detected in human skin by western blotting (not shown).

In human epidermis reconstructed at the air–liquid interface from human keratinocyte primary cultures (**Fig 1*c***, *lane 1*), the 30–35 kDa band of glycosylated AQP3 was also abundant. A band of ≈50 kDa was also detected, which could represent unglycosylated AQP3 dimers. Indeed, in reconstructed epidermis, both species (30–35 kDa and 50 kDa) were specific for the antigenic peptide (**Fig 1*c***, *lane 2*) and for anti-AQP3 antibodies (**Fig 1*c***, *lanes 3*). AQP3 in reconstructed epidermis was strongly detected and showed little change in the intensity of staining from day 0 up to 4 wk of culture at the air–liquid interface (not shown).

Altogether, these results suggest that AQP3 is abundant in native human epidermis and expressed early in human epidermis reconstructed in primary cultures.

Indirect immunofluorescence By indirect immunofluorescence, on 5 μm human skin cryosections, polyclonal anti-AQP3 antibodies strongly stained keratinocyte plasma membranes (**Figs 2*a***, **3*a***). The SC (*arrows*) and the dermis were unstained. In human skin epidermis, staining for AQP3 consistently decreased sharply one layer of cells below the SC. No staining was observed when the antibodies were incubated in the presence of the AQP3 C-terminus peptide used for immunization (**Fig 2*b***). In addition to plasma membrane staining, intracellular staining for AQP3 was often detected in cells at the basal layer of the epidermis (**Fig 2*a***, *arrowheads*). By double staining with anti-AQP3 and anti-keratin 14 (a marker of basal cells) antibodies (**Fig 3*a***, ***b***), cells where intracellular labeling for AQP3 was detected (**Fig 3*a***) were also positive for keratin 14 (**Fig 3*b***), confirming that these were basal cells. The intracellular localization of AQP3 in these cells was confirmed at the electron microscope level (**Fig 3*c***). Accordingly, some basal cells exhibited abundant gold labeling for AQP3 (**Fig 3*c***, *arrows*) but little plasma membrane labeling.

In reconstructed human epidermis, as in native epidermis, anti-AQP3 antibodies strongly stained cell plasma membranes in viable layers of reconstructed epidermis (**Fig 4*a***). As in native skin, staining for AQP3 decreased abruptly in the last layer of granular cells below the SC (**Fig 4**, *arrows*) and no signal was observed in the presence of the antigen (**Fig 4*b***).

Electron microscope gold labeling The cellular and subcellular localization of AQP3 in human keratinocyte plasma membranes was further investigated at the electron microscope level. By gold labeling on human epidermis ultrathin sections, no labeling for AQP3 was detected in the SC (**Fig 5*b***) or when the antibodies were incubated in the presence of the peptide used for immunization (**Fig 5*d***). In contrast, in keratinocyte cells, affinity-purified antibodies against AQP3 exclusively labeled the plasma membrane (**Fig 5*a***, ***c***). AQP3 membrane labeling was always detected all around cells and on both membranes of adjacent keratinocytes (**Fig 5*a***, ***b***), suggesting that this protein is not polarized in keratinocytes. Finally, in basal cells of the human epidermis, plasma membrane labeling was weaker, but an abundant intracellular labeling for AQP3 was often detected (**Fig 3*c***).

Osmotic water fluxes Osmotically induced water fluxes were recorded across the epidermis of human skin fragments and reconstructed epidermis. Water flux measurement in reconstructed epidermis, measured in the presence of a 150 mOsm osmotic gradient, showed a progressive decrease of transepidermal water permeability as the time of culture at the air–liquid interface (and the thickness of the tissue) increased

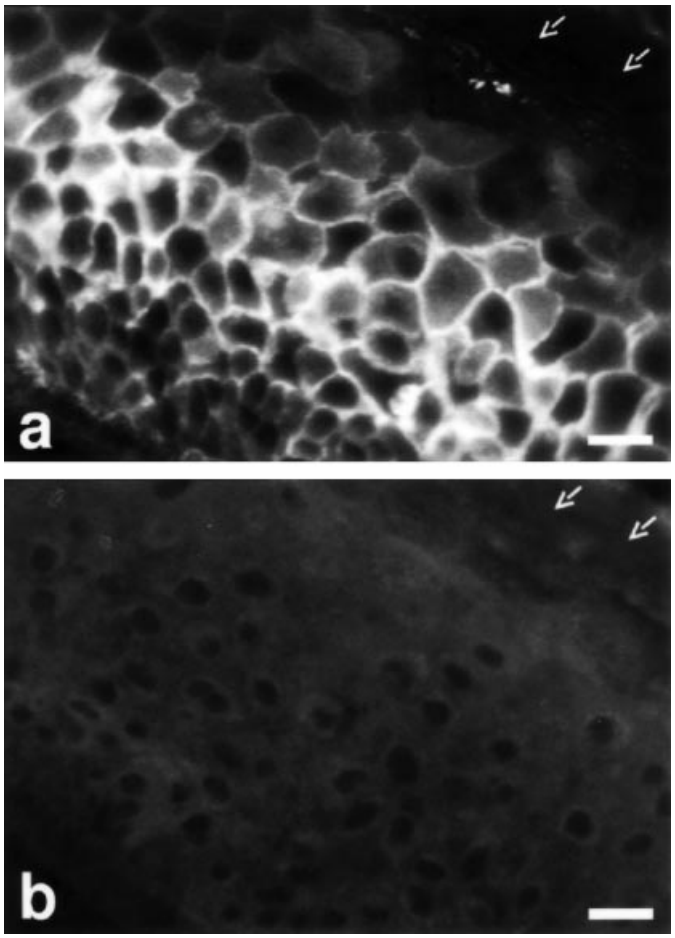

Figure 4. Immunofluorescence localization of AQP3 in reconstructed epidermis. On 5 μm reconstructed epidermis cryosections, polyclonal anti-AQP3 antibodies strongly stained basal and intermediate layers (*a*). The SC (*arrows*) was unstained. No staining was observed when antibodies were incubated in the presence of the AQP3 C-ter peptide used for immunization (*b*). *Scale bar*: 20 μm.

(**Fig 6*a***, *full bars*; 11 d, 19 d, and 24 d shown). No water movement was detected in the absence of an osmotic gradient (not shown). 1 mM $HgCl_2$, a classical inhibitor of aquaporins including AQP3 (Kuwahara *et al*, 1997), induced a marked and significant inhibition (≈70%, $p < 0.05$, $n = 4$; **Fig 6*a***, *empty bar*, 19 d) of osmotic water fluxes. This inhibition was largely reversed after the addition of 5 mM β-mercaptoethanol (**Fig 6*a***, *hashed bar*). As expected, no osmotic water flux was detected in intact human skin epidermis. On skin fragments from which the SC had been stripped, however, small but significant water fluxes could be measured. In the same experimental conditions as with reconstructed epidermis, a water flux of 0.16 ± 0.03 μl per min per cm^2 ($n = 3$) was achieved (**Fig 6*b***, *full bar*). This value is comparable to reconstructed epidermis between day 11 and day 19 of culture at the air–liquid interface (0.17 ± 0.05 and 0.11 ± 0.02 μl per min per cm^2, respectively, $n = 4$). Osmotic water fluxes in human epidermis were decreased by ≈50% after treatment with 1 mM $HgCl_2$. In native human skin, however, 5 mM β-mercaptoethanol failed to reverse completely the mercury inhibition of water permeability and the reversal was slow. We hypothesize that impaired diffusion of both the mercury inhibitor and the reducing agent could account for the smaller effects observed in native human epidermis.

AQP3-mediated water transport was recently reported to be pH sensitive (Zeuthen and Klaerke, 1999). Therefore, the effect of pH on osmotic water fluxes was also examined in reconstructed epidermis. **Figure 7(*a*)** shows a representative experiment on a reconstructed epidermis grown for 17 d at the air–liquid interface. A 150 mOsm osmotic gradient induced a sustained water transport of ≈0.12 μl per min per cm^2. When the pH of the solution bathing the preparation was switched from 7 to 4 (by addition of HCl) the osmotic water flux rapidly dropped to become undetectable. This effect was fully reversed by submitting the preparation to an alkaline pH (by addition of NaOH to the bath). **Figure 7(*b*)** provides a summary of these results and shows a significant inhibition of water fluxes from 0.15 ± 0.02 μl per min per cm^2 to 0.04 ± 0.02 μl per min per cm^2 ($n = 6$) by acid pH, fully reversed to 0.18 ± 0.04 μl per min per cm^2 by alkaline pH.

Keratinocyte osmotic water permeability The osmotic water permeability of keratinocyte membrane vesicles from human epidermis was determined by stopped-flow light scattering. **Figure 8** (*left*) shows the average time course of keratinocyte plasma membrane vesicle shrinkage, detected by the intensity of light scattering at 90° (see Roudier *et al*, 1998), in response to a 250 mOsm osmotic shock. The average P_f at pH 7 was 1.7×10^{-2} cm per s. When membrane vesicles were incubated at pH 5, the P_f was decreased 2.5-fold to 0.67×10^{-2} cm per s. Western blotting with anti-AQP3 antibodies confirmed the presence of AQP3 in membrane vesicles (**Fig 8**, *right*). These results demonstrate a high, pH-sensitive, water permeability for keratinocyte plasma membranes, consistent with the presence of functional AQP3 in these membranes.

DISCUSSION

Aquaporins are molecular transmembrane water channels expressed in many epithelial and endothelial cells, where they facilitate water transport along a favorable osmotic gradient (van Os *et al*, 2000; Verkman *et al*, 2000). Over the past few years, the physiologic role of aquaporins has been well studied in kidney tubules, where water reabsorption plays a critical role in the concentration of urine (Deen *et al*, 1994; Chou *et al*, 1998; Ma *et al*, 1998, 2000; Verkman, 1998; van Os *et al*, 2000). Similarly, the role of aquaporins in the production of cerebrospinal fluid in brain is currently being investigated (Manley *et al*, 2000; Masseguin *et al*, 2000). Aquaporins were also identified in the reproductive tract, in exocrine glands (van Os *et al*, 2000; Steward and Kwon, 2000), and in the gastrointestinal tract, where solute and water transport have long been known to occur. Nevertheless, aquaporins were also identified in other tissues, like skin, not generally believed to be primarily involved in fluid transports and where their role remains to be elucidated. Perhaps because water movements in the skin epidermis were not thought to play an important physiologic role, the expression of aquaporins in this tissue has not been thoroughly investigated, although AQP3 was identified in rat epidermis shortly after it was cloned (Frigeri *et al*, 1995). Instead, aquaporins in skin were actively searched in sweat glands, without success to date. Nevertheless, the skin epidermis is a highly organized multilayered epithelium, where water and solute transport play a critical role in the formation of an impermeable barrier at the SC level (Warner *et al*, 1988). Unlike monolayered transporting epithelia, however, fluid transport in complex, multilayered, epidermis cannot be assessed easily. Nevertheless, this does not exclude the hypothesis that solute transport could induce water movements involving aquaporins across the viable layers of this tissue.

The purpose of this work was to examine the presence of aquaporins in human epidermis and to determine whether these channels could play a significant physiologic role in this tissue. Aquaporin expression and function were also investigated in reconstructed human epidermis, used as an *in vitro* model of human skin.

AQP3 expression and localization By indirect immunofluorescence and electron microscopy gold labeling on human epidermis sections, AQP3 was primarily and abundantly localized in keratinocyte plasma membranes. This is consistent with the high

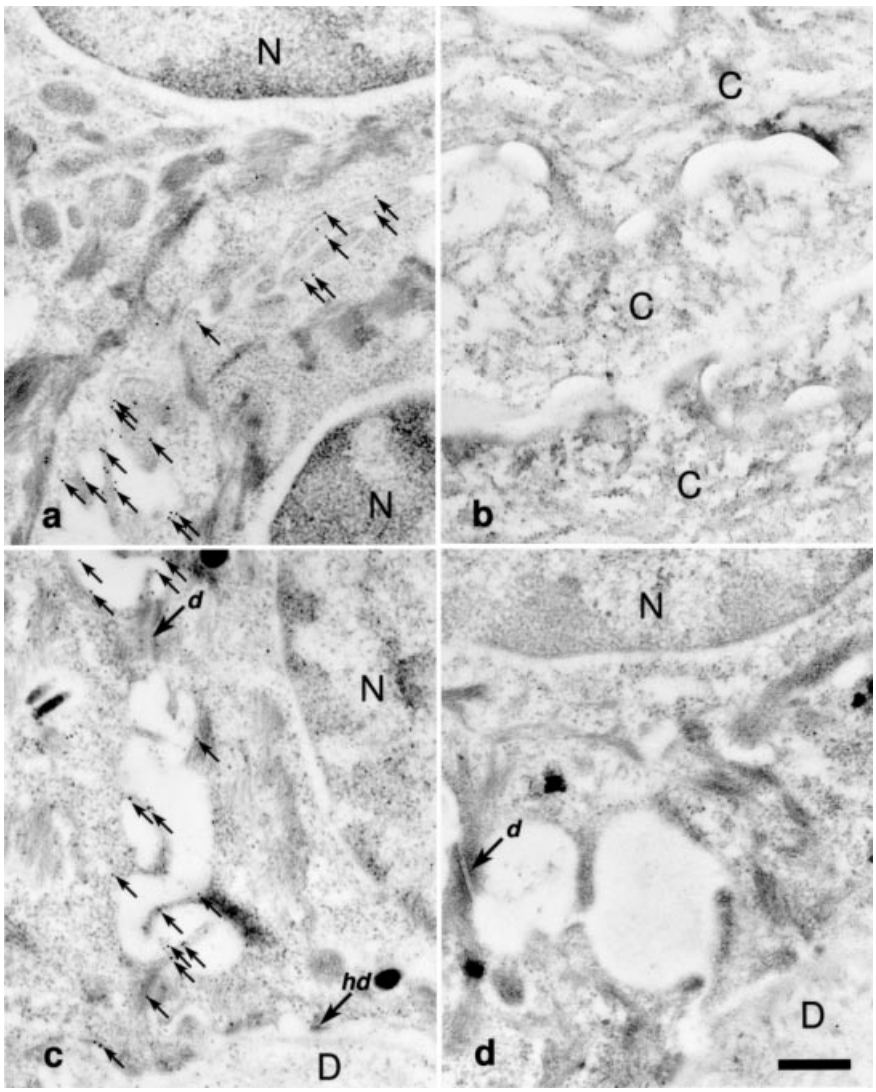

Figure 5. Electron microscope gold localization of AQP3 in human epidermis. On thin sections of human epidermis, no gold labeling for AQP3 with affinity-purified antibodies was detected in the SC (*b*) or when antibodies were preabsorbed with the immune peptide (*d*). In keratinocytes, gold labeling (*arrows*) was mainly localized to the plasma membrane (*a*, *c*). C, corneocyte; D, dermis; N, nucleus. *Scale bar*: 250 nm.

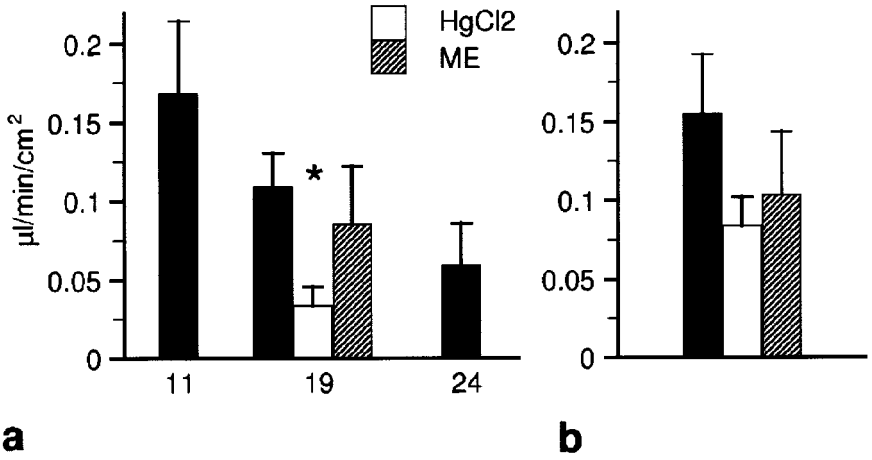

Figure 6. Osmotic water fluxes across human epidermis. (*a*) Water transport across reconstructed epidermis along a 150 mOsm osmotic gradient decreased with time of culture (11, 19, 24 d, *full bars*). Water movements were significantly inhibited by ≈70% by 1 mM $HgCl_2$ (*empty bar*). This inhibition was mostly reversed by 5 mM β-mercaptoethanol (ME, *hashed bars*). n = 4. (*b*) In stripped, native human epidermis, osmotic water transport was comparable to that in reconstructed epidermis (*full bar*, n = 3). In human epidermis, however, inhibition of water movements by 1 mM $HgCl_2$ (≈50%, *empty bar*) and its reversal by 5 mM β-mercaptoethanol (*hashed bar*) were incomplete, perhaps because of the increased complexity of this tissue.

level of expression of this protein in human epidermis as found by western blotting, as keratinocytes are the major cell type of this tissue. The plasma membrane localization of AQP3 in these cells is also consistent with its localization in other cell types. Completely unstained cells were never observed in viable cells of the epidermis, but the absence of AQP3 in other cell types of the epidermis cannot be excluded because of the presence of adjacent keratinocytes. At the electron microscope level, plasma membrane labeling for AQP3 was detected all around cells, suggesting the absence of AQP3 polarity in this tissue, in contrast to epithelial cells with tight junctions like kidney-collecting-duct principal cells. Interestingly, intracellular AQP3 was also detected in the less differentiated basal layer of the epidermis, possibly suggesting that AQP3 targeting can be regulated *in vivo*. AQP3 synthesis appears to occur early in basal

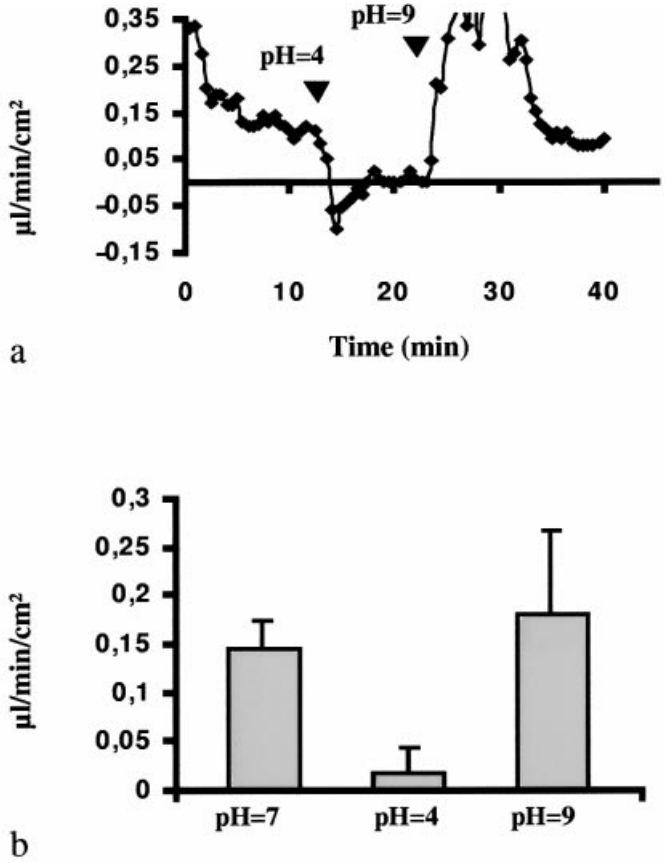

Figure 7. pH-sensitive water fluxes in reconstructed epidermis. (*a*) A typical continuous measurement of water movements across reconstructed epidermis in the presence of a 150 mOsm osmotic gradient. After stabilization of the preparation (less than 5 min), a sustained ≈0.12 µl per min per cm^2 water flux is detected at pH 7.4. Acidification of the preparation (pH 4) induces a rapid and complete inhibition of water movements, despite the osmotic gradient. Following another change in pH (pH 9), water movements rapidly increase and after a few minutes are restored to their initial value. (*b*) In six experiments, the average water flux at pH 7.4 (0.15 µl per min per cm^2), was significantly ($p < 0.01$) inhibited to 0.04 µl per min per cm^2 at acid pH. Reversal of water fluxes by alkaline pH to 0.19 µl per min per cm^2 was significant ($p < 0.01$), but not different from the initial value ($p > 0.3$).

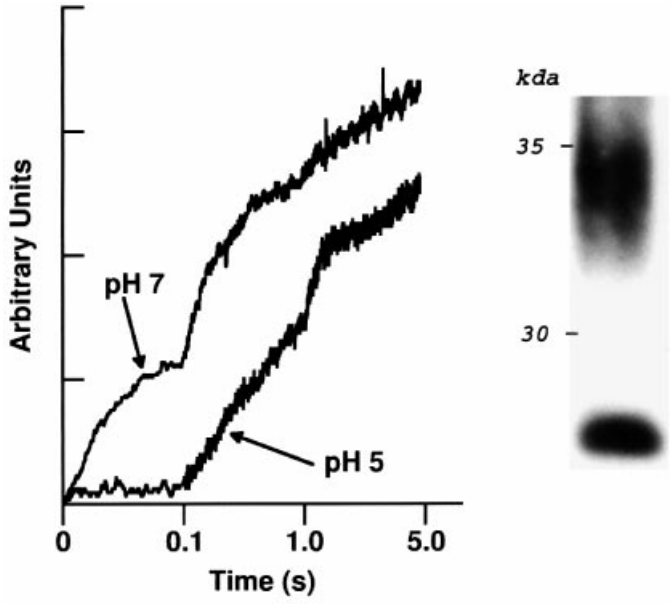

Figure 8. Stopped-flow light scattering in keratinocyte membrane vesicles. By western blotting with anti-AQP3 antibodies, membrane vesicles from keratinocytes isolated from human epidermis showed a strong signal for AQP3 (*right*). *Left* shows the average time course of membrane vesicle shrinkage in response to a 250 mOsm gradient ($n = 3$) at pH 7 and pH 5 measured by stopped-flow light scattering. Human keratinocyte membrane vesicles showed a high water permeability ($P_f = 1.7 \times 10^{-2}$ cm s^{-1}) at pH 7. This permeability was reduced at pH 5 ($P_f = 0.67 \times 10^{-2}$ cm s^{-1}).

cells with a predominant cytoplasmic distribution and the differentiation process could induce AQP3 translocation to the plasma membrane. As expected, no AQP3 was detected at the highly water impermeable SC. The presence of an aquaporin in keratinocyte plasma membranes in viable layers of the skin epidermis suggests that it could play a role in water transport within this tissue. The cellular localization of AQP3 in reconstructed epidermis was comparable to that of the native form in intact skin, which makes this *in vitro* model a valuable tool for physiologic studies. No aquaporin expression was detected in skin dermis, where water can diffuse rapidly because of its loose structure and the presence of highly hydrated glycosaminoglycans. Finally, AQP1, AQP2, AQP4, AQP5 were not detected in human epidermis at the protein level. This does not exclude the presence of low levels of these species or of other aquaporin isoforms, but the abundance of AQP3 alone could be sufficient to confer a high permeability to keratinocytes.

Water transport and water permeability in the epidermis Consistent with its barrier function, the water permeability of intact human skin was not measurable. After stripping, however, detectable water fluxes were observed across viable layers of native human skin epidermis along a favorable osmotic gradient. Because of the large thickness of this tissue compared to epithelial monolayers, this suggests that the water permeability of each layer of the epidermis, below the SC, may be remarkably higher. Functional studies on reconstructed epidermis suggest that this model recapitulates water transport in native skin epidermis. Water transport in the epidermis was mercury- and pH-sensitive, consistent with the biophysical properties of AQP3 (Kuwahara *et al*, 1997; Zeuthen and Klaerke, 1999), and water fluxes across stripped epidermis were higher than the transepidermal water loss of intact skin but comparable to the transepidermal water loss of stripped skin (Spruit, 1970). The complex structure of either native or reconstructed epidermis, however, does not allow water permeability coefficients to be deduced from water fluxes (Verkman *et al*, 2000). Therefore the water permeability of keratinocyte plasma membranes was directly studied by stopped-flow light scattering on keratinocyte membrane vesicles. In agreement with AQP3 localization, keratinocyte membrane vesicles exhibited a high, pH-sensitive, water permeability ($P_f = 1.7 \times 10^{-2}$ cm per s), consistent with the involvement of water channels (Verkman *et al*, 2000), further suggesting that AQP3 is functional in this tissue.

Physiologic role of AQP3 in the epidermis As the primary barrier between the environment and the body, the skin epidermis is constitutively exposed to sharp gradients. In particular, a steep water content gradient was found across the SC towards the stratum granulosum, whereas water content was constant deeper in the epidermis (Warner *et al*, 1988). Similarly, whereas the surface pH of skin is around 5, it increases to 7 below the SC (Ohman and Vahlquist, 1994). What then could be the role of a pH-sensitive water channel in this tissue?

The sharp decrease in keratinocyte AQP3 expression immediately below the SC, as well as the inhibition of its water channel function at acid pH, are both in agreement with the function of water loss prevention of the SC. In the absence of blood vessels

within the epidermis, however, the barrier formed by the SC might prevent water from moving from the dermis into living layers of the epidermis. Therefore we suggest that AQP3 provides a short circuit for water, or water-clamp, between the base of the epidermis and the SC, in order to maintain a constant water content and to prevent the formation of a continuous water gradient across the epidermis below the SC. Whereas such a gradient would probably affect the stratification and differentiation process in human epidermis, an AQP3-mediated discontinuity in water permeability between deep epidermis and the SC could account for the observed discontinuity in water contents, which effectively isolates the SC from living tissue and plays an important role in the barrier function of the epidermis and in body water conservation (Warner *et al*, 1988). It should be noted in particular that the site of water partitioning in the epidermis was suggested to be in the last granular cell layer below the SC (Warner *et al*, 1988), which is also where we found AQP3 expression to disappear.

In addition to its water permeability, AQP3 is also permeable to small uncharged solutes such as urea and glycerol (Roudier *et al*, 1998; Zeuthen and Klaerke, 1999; Ma *et al*, 2000). Although the physiologic role of AQP3 in water transport is well established in kidney (Ma *et al*, 2000), small solute transport by AQP3 has been much less studied. Therefore, we cannot exclude an additional role for AQP3 in glycerol or urea transport within the epidermis. The recent generation of transgenic mice lacking AQP3 (Ma *et al*, 2000) could prove useful to investigate the functional role of AQP3 in the epidermis further. Mouse epidermis, however, is several times thinner than human epidermis. Therefore, AQP3 expression may not be as critical in mice as in humans. Alternatively, our results strongly suggest that reconstructed epidermis is a reliable model of human skin epidermis both morphologically and functionally.

In conclusion, our work demonstrates that AQP3 is abundant in native and reconstructed human skin epidermis, where it is primarily localized to keratinocyte plasma membranes. AQP3 distribution in the epidermis exhibits a discontinuity one layer of cells below the SC, consistent with water distribution in this tissue. In addition water transport and water permeability studies demonstrate that AQP3 is functional in human epidermis and confers a high water permeability to viable layers of the epidermis. These results lead us to suggest that AQP3 can play a significant role in the hydration of the epidermis, by preventing the formation of an osmotic gradient across viable layers of this tissue.

This work is dedicated to the memory of Patrick Barré. The work was supported in part by the Europa Union TMR grant ERBFMRXCT970128.

REFERENCES

Bai C, Fukuda N, Song Y, Ma T, Matthay MA, Verkman AS: Lung fluid transport in aquaporin-1 and aquaporin-4 knockout mice. *J Clin Invest* 103:555–561, 1999

Borgnia M, Nielsen S, Engel A, Agre P: Cellular and molecular biology of the aquaporin water channels. *Annu Rev Biochem* 68:425–458, 1999

Bourguet J, Jard S: Un dispositif automatique de mesure et d'enregistrement du flux net d'eau à travers la peau et la vessie desamphibiens. *Biophys Biochim Acta* 88:442–444, 1964

Chou CL, Ma T, Yang B, Knepper MA, Verkman AS: Fourfold reduction of water permeability in inner medullary collecting duct of aquaporin-4 knockout mice. *Am J Physiol* 274:C549–C554, 1998

Deen PM, Verdijk MA, Knoers NV, Wieringa B, Monnens LA, van Os CH, van Oost BA: Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. *Science* 264:92–95, 1994

Dorr RA, Kierbel A, Vera J, Parisi M: A new data-acquisition system for the measurement of the net water flux across epithelia. *Comput Meth Programs Biomed* 53:9–14, 1997

Frigeri A, Gropper MA, Umenishi F, Kawashima M, Brown D, Verkman AS: Localization of MIWC and GLIP water channel homologs in neuromuscular, epithelial and glandular tissues. *J Cell Sci* 108:2993–3002, 1995

Frigeri A, Nicchia GP, Verbavatz JM, Valenti G, Svelto M: Expression of aquaporin-4 in fast-twitch fibers of mammalian skeletal muscle. *J Clin Invest* 102:695–703, 1998

King LS, Choi M, Fernandez PC, Cartron JP, Agre P: Defective urinary-concentrating ability due to a complete deficiency of aquaporin-1. *N Engl J Med* 345:175–179, 2001

Kuwahara M, Gu Y, Ishibashi K, Marumo F, Sasaki S: Mercury-sensitive residues and pore site in AQP3 water channel. *Biochemistry* 36:13973–13978, 1997

Landmann L: The epidermal permeability barrier. *Anat Embryol (Berl)* 178:1–13, 1988

Ma T, Yang B, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS: Severely impaired urinary concentrating ability in transgenic mice lacking aquaporin-1 water channels. *J Biol Chem* 273:4296–4299, 1998

Ma T, Song Y, Yang B, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS: Nephrogenic diabetes insipidus in mice lacking aquaporin-3 water channels. *Proc Natl Acad Sci USA* 97:4386–4391, 2000

Manley GT, Fujimura M, Ma T, et al: Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat Med* 6:159–163, 2000

Masseguin C, Corcoran M, Carcenac C, Daunton NG, Guell A, Verkman AS, Gabrion J: Altered gravity downregulates aquaporin-1 protein expression in choroid plexus. *J Appl Physiol* 88:843–850, 2000

Mathai JC, Mori S, Smith BL, et al: Functional analysis of aquaporin-1 deficient red cells. The Colton-null phenotype. *J Biol Chem* 271:1309–1313, 1996

Matsuzaki T, Suzuki T, Koyama H, Tanaka S, Takata K: Water channel protein AQP3 is present in epithelia exposed to the environment of possible water loss. *J Histochem Cytochem* 47:1275–1286, 1999

Murata K, Mitsuoka K, Hirai T, et al: Structural determinants of water permeation through aquaporin-1. *Nature* 407:599–605, 2000

Nielsen S, Smith BL, Christensen EI, Agre P: Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. *Proc Natl Acad Sci USA* 190:7275–7279, 1993a

Nielsen S, Smith BL, Christensen EI, Knepper MA, Agre P: CHIP28 water channels are localized in constitutively water-permeable segments of the nephron. *J Cell Biol* 120:371–383, 1993b

Noel-Hudson MS, Dusser J, Collober J, *et al*: Human epidermis reconstructed on synthetic membrane: influence of experimental conditions on terminal differentiation. *In Vitro Cell Dev Biol Anim* 31:508–515, 1995

Ohman H, Vahlquist A: *In vivo* studies concerning a pH gradient in human stratum corneum and upper epidermis. *Acta Derm Venereol* 74:375–379, 1994

van Os CH, Kamsteeg EJ, Marr N, Deen PM: Phsyiological relevance of aquaporins: luxury or necessity? *Pflugers Arch* 440:513–520, 2000

Preston GM, Carroll TP, Guggino WB, Agre P: Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science* 256:385–387, 1992

Ren G, Cheng A, Reddy V, Melnyk P, Mitra AK: Three-dimensional fold of the human AQP1 water channel determined at 4 A resolution by electron crystallography of two-dimensional crystals embedded in ice. *J Mol Biol* 301:369–387, 2000

Roudier N, Verbavatz JM, Maurel C, Ripoche P, Tacnet F: Evidence for the presence of aquaporin-3 in human red blood cells. *J Biol Chem* 273:8407–8412, 1998

Sabolic I, Valenti G, Verbavatz JM, Van Hoek AN, Verkman AS, Ausiello DA, Brown D: Localization of the CHIP28 water channel in rat kidney. *Am J Physiol* 263:C1225–C1233, 1992

Shi LB, Skach WR, Verkman AS: Functional independence of monomeric CHIP28 water channels revealed by expression of wild-type mutant heterodimers. *J Biol Chem* 269:10417–10422, 1994

Spruit D: The water barrier of stripped and normal skin. *Dermatologica* 141:54–59, 1970

Steward M, Kwon TH: Aquaporin water channels in exocrine glands. *J Korean Med Sci* 15:S7–S9, 2000

Toriano R, Ford P, Rivarola V, Tamarappoo BK, Verkman AS, Parisi M: Reconstitution of a regulated transepithelial water pathway in cells transfected with AQP2 and an AQP1/AQP2 hybrid containing the AQP2-C terminus. *J Membr Biol* 161:141–149, 1998

Verbavatz JM, Brown D, Sabolic I, *et al*: Tetrameric assembly of CHIP28 water channels in liposomes and cell membranes: a freeze-fracture study. *J Cell Biol* 123:605–618, 1993

Verkman AS: Role of aquaporin water channels in kidney and lung. *Am J Med Sci* 316:310–320, 1998

Verkman AS, Mitra AK: Structure and function of aquaporin water channels. *Am J Physiol Renal Physiol* 278:F13–F28, 2000

Warner RR, Myers MC, Taylor DA: Electron probe analysis of human skin: determination of the water concentration profile. *J Invest Dermatol* 90:218–224, 1988

Yang B, Verbavatz JM, Song Y, et al: Skeletal muscle function and water permeability in aquaporin-4 deficient mice. *Am J Physiol Cell Physiol* 278:C1108–C1115, 2000

Zeuthen T, Klaerke DA: Transport of water and glycerol in aquaporin 3 is gated by H(+). *J Biol Chem* 274:21631–21636, 1999