Aquaporin 2 Promotes Cell Migration and Epithelial Morphogenesis

Ying Chen,* William Rice,* Zhizhan Gu,†‡ Jian Li,†‡ Jianmin Huang,* Michael B. Brenner,†‡ Alfred Van Hoek,* Jianping Xiong,* Gregg G. Gundersen,§ Jim C. Norman,‖ Victor W. Hsu,‖ Robert A. Fenton,¶ Dennis Brown,* and Hua A. Jenny Lu*

*Center for Systems Biology, Program in Membrane Biology and Division of Nephrology, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Simches Research Center, Boston, Massachusetts; †Division of Rheumatology, Immunology, and Allergy, Brigham and Women’s Hospital, Boston, Massachusetts; ‡Department of Medicine, Harvard Medical School, Boston, Massachusetts; §Department of Pathology and Cell Biology, Columbia University, New York, New York; ‖Integrin Cell Biology Laboratory, Beatson Institute for Cancer Research, Glasgow, Scotland; and ¶Water and Salt Research Center, Department of Anatomy, Aarhus University, Aarhus, Denmark

ABSTRACT

The aquaporin 2 (AQP2) water channel, expressed in kidney collecting ducts, contributes critically to water homeostasis in mammals. Animals lacking or having significantly reduced levels of AQP2, however, have not only urinary concentrating abnormalities but also renal tubular defects that lead to neonatal mortality from renal failure. Here, we show that AQP2 is not only a water channel but also an integrin-binding membrane protein that promotes cell migration and epithelial morphogenesis. AQP2 expression modulates the trafficking and internalization of integrin β1, facilitating its turnover at focal adhesions. In vitro, disturbing the interaction between AQP2 and integrin β1 by mutating the RGD motif led to reduced endocytosis, retention of integrin β1 at the cell surface, and defective cell migration and tubulogenesis. Similarly, in vivo, AQP2-null mice exhibited significant retention of integrin β1 at the basolateral membrane and had tubular abnormalities. In summary, these data suggest that the water channel AQP2 interacts with integrins to promote renal epithelial cell migration, contributing to the structural and functional integrity of the mammalian kidney.


Aquaporin 2 (AQP2) is a water channel that mediates water absorption through the apical membrane of the principal cells of the kidney collecting ducts, and contributes critically to water homeostasis in mammals. Under physiologic conditions, the trafficking of AQP2 is regulated mainly by vasopressin via a well established signaling pathway that results in the elevation of cAMP, activation of protein kinase A (PKA) and phosphorylation of AQP2.1–3 In addition to regulated trafficking, AQP2 also constitutively recycles between intracellular vesicles and the cell membrane.4,5 Critical protein/protein interactions that orchestrate the regulated and constitutive trafficking of AQP2 have been extensively investigated and include actin and microtubules, SNAREs, Rab proteins, heat shock protein 70, clathrin, and others.6–9 However, emerging data from AQP2 knockout and transgenic animals suggested an unusual aspect of AQP2 biology. As expected, induction of AQP2 deficiency in mice results in a urinary concentrating defect known as diabetes insipidus (DI). However, these animals also presented with neonatal mortality from renal failure with renal tubular abnormalities.10–12 This striking phenotype was thought to result from their profound polyuria. However, mice

Received January 24, 2012. Accepted May 30, 2012.

Published online ahead of print. Publication date available at www.jasn.org

Correspondence: Dr. Hua A. Jenny Lu, Center for Systems Biology, Program in Membrane Biology and Division of Nephrology, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Simches Research Center, 185 Cambridge Street, Boston, MA 02114. Email: halu@partners.org

Copyright © 2012 by the American Society of Nephrology
lacking AQP1, AQP3, or AQP4, which manifest similar concentrating defects/DI, do not have renal failure or reduced survival. Similarly, although AQP2 protein is preserved in the vasopressin-deficient Brattleboro rat (with spontaneous mutation of the vasopressin gene leading to “central” DI) or mice with overactive cAMP phosphodiesterase in principal cells, both have polyuria from birth without tubular/renal malformation or kidney failure. Interestingly, several other mouse models in which polyuria is associated with perturbations in renal structure are reported to have greatly reduced AQP2 expression, whereas polyuric models with no structural abnormalities have normal AQP2 levels. Thus, we hypothesized that the tubular abnormalities seen in the neonatal AQP2 knockout animals and/or AQP2 transgenics are due to an intrinsic defect associated with the absence or dysfunction of AQP2 rather than being a secondary effect of polyuria.

We identified an Arg-Gly-Asp (RGD) potential integrin-binding motif in the second external loop of AQP2, and our data suggested an interaction of AQP2 and integrins. Interestingly, it was recently reported by an independent group that integrin signaling modulates the trafficking of AQP2 through interacting with AQP2 via its RGD motif. Integrins are a large family of cell surface receptors that mediate heterophilic cell-cell and cell-extracellular matrix (ECM) interactions. They are widely expressed in ureteric bud derivatives and collecting duct epithelial cells in developing kidney as well as mature tubules, contributing critically to kidney development and repair. Integrins consist of an α and β chain that form an interface for recognizing and binding an “RGD” motif (or other sequence) that is present in ECM components. Upon activation, integrins recruit multiple cellular proteins forming focal adhesions (FAs). The internalization and/or recycling of integrins affects the formation and disassembly of FAs, and the formation and turnover of FAs is in turn a key factor determining the rate and direction of cell migration. Among many types of integrins, integrin β1 is the most abundant subunit that is expressed in almost all organs and all cell types, especially in the kidney. Work on integrin β1 knockout mice shows that it plays a critical role in kidney collecting duct development and maintaining tubular integrity in the developed kidney.

Here we report an association of a water channel, AQP2, with integrin β1. Via this interaction, AQP2 modulates the intracellular trafficking and subsequent surface presentation of integrin β1, and thereby modulates epithelial cellular events mediated by integrin β1 that ultimately contribute to kidney epithelial morphogenesis.

RESULTS

Kidney Tubular Defects in AQP2-Deficient Mice

Re-examination of kidneys from adult (5 weeks) and younger (postnatal day 7) AQP2 knockout mice showed significant

Figure 1. Tubular abnormalities and altered distribution of integrin β1 in AQP2 knockout animals. (A) Gross abnormalities in kidney structure in AQP2 knockout animals 5 weeks after birth. (B) Tubular dilation and microcyst formation (arrows) in AQP2 knockout animals at postnatal day 7. AQP4 (green) indicates the tubules are collecting ducts. (C) Altered distribution of integrin β1 in collecting ducts of AQP2 knockout animals. Integrin β1 (red) is in the subbasal membrane domain in wild-type (control) animals, but is concentrated on the lateral membrane in knockout mice. The subcellular distribution of β-catenin (green) is not altered. KO, knockout; L, lumen. Scale bar, 1 mm in A; 50 μm in B; 10 μm in C.
AQP2 Promotes Kidney Epithelial Cell Migration

To investigate the functional significance of AQP2 and integrin interaction, a panel of in vitro assays was applied to kidney epithelial MDCK cells as well as LLCPK1 cells. RT-PCR and enhanced immunoblot detection revealed the presence of a low level of endogenous AQP2 in the parental MDCK cells, which were considered previously as “AQP2 null” cells (Figure 3A). All of the assays were performed in both MDCK and LLCPK1 cells stably expressing rat AQP2 and its RGD/A mutant. Overexpression of AQP2 or AQP2 RGD/A mutant does not cause any significant change in the expression of integrins α2, α5, and β1 in these cells (Figure 3A). Although no significant differences in cell proliferation and apoptosis were detected, a striking difference in cell migration and tubulogenesis was found among these cells.

Epithelial cell migration is a fundamental process during kidney embryogenesis and tissue remodeling after injury. Interestingly, we observed a polarized expression of AQP2 in the lamellipodia/leading edge in migrating MDCK-AQP2 cells where it colocalized with integrin β1 (Figure 3B). A wound closure assay was applied to untransfected MDCK cells and cells expressing wild-type AQP2 and AQP2 RGD/A. Expression of AQP2 accelerated wound closure on a fibronectin-coated surface compared with untransfected cells, whereas expression of the AQP2-RGD/A mutant significantly inhibited wound closure (Figure 3C). The rate of migration of cells toward the wounded margin was calculated (Figure 3C). This promigratory effect of AQP2 was also observed in transfected LLCPK1 cells to a similar degree. The migration of AQP2 transfected and untransfected LLCPK1 cells on fibronectin-coated plates was revealed by time-lapse imaging and a more coordinated cell migration toward the wounded region was seen (Supplemental Movies 1–3). However, this promigratory effect of AQP2 was not observed on a collagen type I–coated surface (Supplemental Figure 4), suggesting that AQP2 requires specific ECM component(s) or ECM/integrin signaling for its promigratory effect. Moreover, this promigratory effect was not observed in cells transfected with AQP0, a water channel that shares the most homology with AQP2 in the aquaporin family, but containing an RGN sequence instead of the RGD motif attuned to the interaction of AQP2 and integrin β1 (Figure 2D).

Supporting AQP2 and integrin interaction, our detailed examination revealed the presence of basolaterally located AQP2 in the same subcellular domain as integrin β1 in the principal cells of the collecting ducts from adult rats (Figure 2E). Integrins are predominantly located in the basal membrane and subbasal domains in kidney tubular cells, whereas AQP2 is a well-known apically functional membrane channel. Despite this widely accepted concept, it has been observed for many years that there is a significant amount of AQP2 located in the basolateral domain in many principal cells, which is in agreement with our data.33–35
Figure 2. AQP2 interacts with integrins via an RGD motif. (A) AQP2 and integrin β1 coimmunoprecipitate (co-IP) from rat kidney (RK) and cell lysates (from stable LLCPK1 cells expressing wild-type AQP2), using anti-AQP2 (left) and anti-integrin β1 (right) antibodies for IP. Co-IP was also performed in vasopressin-stimulated cells (vasopressin). The presence of AQP2 or integrin β1 in corresponding co-IP complex is shown in the lower panel. (B) AQP2 interacts with integrin α5, but not integrin α2 by co-IP using RK lysate. Integrin β1 is present in the co-IP complex using integrin α2, α5, and β1 antibodies for IP and is shown in lower panel. (C) Synthetic AQP2 peptide attenuates the interaction of AQP2 and integrin β1, and AQP2 and integrin α5. (D) Mutation of the AQP2 RGD motif (lane 3, AQP2 RGD/A-expressing LLCPK1 cells) attenuates AQP2 and integrin β1 interaction. Data are representative of at least three experiments. “B” indicates beads only. (E) Immunofluorescence staining reveals the co-presence of AQP2 (green, arrow) and integrin β1 signal (red) in the basal membrane domain in tubular cells of the collecting ducts in RK. The insert in right panel highlights the colocalization of AQP2 and integrin β1 on the basal membrane of the AQP2-expressing principal cells. Scale bar, 10 μm.
after forskolin stimulation in both wild-type AQP2 and RGD/A-expressing MDCK cells (Supplemental Figure 5, B and C). Using a 3D-culture tubulogenesis assay, we found that expression of AQP2 in LLCPK1 cells promoted the formation of larger and more complex (branched) tubule-like structures compared with untransfected cells in collagen gel. Remarkably, AQP2 RGD/A-expressing cells not only failed to form tubules, but organized into cyst-like structures (Supplemental Figure 6A). Quantification showed that AQP2 significantly promoted, whereas AQP2 RGD/A significantly inhibited tubulogenesis (Supplemental Figure 6A). A similar effect of AQP2 on tubulogenesis was found in 3D cultures using collagen gel mixed with matrigel (Supplemental Figure 6B). However, tubules formed much faster, taking only 3 days compared with the typical 7-day period to form tubules in pure collagen gels, suggesting that the protubulogenic effect of AQP2 is also subject to modification by various ECM components.36

AQP2 Associates with Integrin β1 and Affects the Trafficking of Integrin β1

Because dynamic trafficking of integrin β1 is critical for many of its associated functions, especially during cell migration,37,38 we postulated that through physical interaction, AQP2 affects the trafficking and subcellular distribution of integrin β1.

Figure 3. AQP2 promotes kidney epithelial cell migration. (A) The expression of endogenous canine AQP2 in untransfected MDCK cells is detected by RT-PCR using dog specific AQP2 primer (dAQP2) and the expression of transfected rat AQP2 is detected by RT-PCR using rat specific AQP2 primer (rAQP2). The presence of endogenous AQP2 in MDCK cells is also detected by immunoblot after overloading the protein lysates of MDCK cells. The expression of integrins β1, α2, and α5 is equal among these cells. (B) AQP2 (green) is polarized to the lamellipodia/leading edge and colocalizes with integrin β1 (red) in the lamellipodia (arrow head) in migrating MDCK cells expressing wild-type AQP2 (upper panel). However, AQP2 does not polarize to the lamellipodia and neither does it colocalize with integrin β1 in the lamellipodia in MDCK cells expressing AQP2 RGD/A mutant (lower panel). Arrow indicates the direction of cell migration. (C) Expression of AQP2 (middle panel) promotes, whereas expression of AQP2 RGD/A inhibits cell migration in a wound-healing assay on fibronectin-coated surface compared with untransfected MDCK cells. The average speed of cell migration over 15 hours was calculated and shown in the right penal. Values are mean ± SEM, n=3, *P<0.05, **P<0.01. Scale bar, 5 μm in B.
Endocytosis is a critical process modulating surface presentation of cell adhesion molecules such as integrins. We first examined the internalization of surface-labeled integrin β1 (with anti-integrin β1 antibody Ts2/16) in the presence or absence of AQP2, or AQP2-RGD/A using a well established endocytosis assay. In this assay, cells were labeled with antibody at 4°C and internalization of the surface-labeled integrin occurred after changing temperature from 4°C to 37°C. Internalized integrin β1 accumulated inside the cell, forming a perinuclear patch, which reached a steady state 2 hours after internalization. Importantly, there is no significant recycling of internalized integrin β1 under this serum-free condition. Expression of wild-type AQP2 in MDCK cells significantly increased the internalization of surface-labeled integrin β1 compared with untransfected cells (containing a low level of endogenous AQP2) (Figure 4A). The ratio of membrane versus total fluorescence signal of integrin β1 and shows a significant cell surface retention of integrin β1 in untransfected cells compared with AQP2-expressing cells at 60 and 120 minutes of internalization (Figure 4B). Endocytosis of integrin β1 was significantly reduced and a dramatic membrane accumulation of integrin β1 occurred in the presence of AQP2 RGD/A, showing a dominant negative effect on integrin internalization (Figure 4, A and B). We next examined the association of AQP2 and integrin β1 during vesicular trafficking. The association of AQP2 and integrins including integrin β1 was first reported in a proteomic analysis of isolated AQP2-containing vesicles. Consistent with this report, internalized, surface-labeled integrin β1 (by Ts2/16 antibody) was detected in vesicles containing clathrin, Rab11, and AQP2 by immunofluorescence staining (Figure 4C), revealing a spatial association of AQP2 and integrin β1 during integrin trafficking.

Therefore, we believe that association of AQP2 and integrin β1 is necessary for “normal” trafficking of integrin β1 in these cells. Loss of this association, or uncoupling, as seen in AQP2 RGD/A mutant, may lead to an overwhelming membrane accumulation of integrin β1 in the “mutant” cells. Indeed, similar to the increased lateral integrin staining in AQP2 null mice, we observed increased basolateral membrane accumulation of integrin β1 in untransfected, polarized MDCK cells and more significantly, in AQP2 RGD/A-expressing cells compared with cells expressing wild-type AQP2 by immunofluorescence staining (Figure 5A), a similar dominant negative effect to that previously observed in the integrin internalization study (Figure 4, A and B). Cell surface accumulation of integrin β1 was further confirmed by surface biotinylation (Figure 5, B and C). An increased surface presentation of integrin β1 and α5 was detected in untransfected cells and AQP2 RGD/A-expressing cells compared with cells expressing wild-type AQP2. This AQP2-dependent membrane accumulation was not seen for integrin α2 or transferrin receptor. To summarize, our data consistently showed that wild-type AQP2 promotes the internalization of integrin β1 and reduces its surface accumulation, whereas the absence of AQP2 or expression of the AQP2 RGD/A mutant leads to membrane

Figure 4. AQP2 modulates the trafficking and subcellular distribution of integrin β1. (A) Expressing wild-type AQP2 in MDCK cells facilitates, whereas a lack of AQP2 expression or expression of AQP2 RGD/A inhibits the internalization of surface-labeled integrin β1 (red) over time. (B) Ratios of membrane retained integrin β1 signal over total integrin β1 signal (membrane and cytosol) after integrin β1 internalization over time were plotted. Values represent the mean ± SEM from at least 50 cells at each time point from three independent experiments. Membrane retention of surface-labeled integrin β1 was significant at 60 and 120 minutes after internalization in untransfected MDCK cells compared with cells expressing wild-type AQP2. A more dramatic membrane accumulation is seen in MDCK cells overexpressing AQP2 RGD/A. (C) Overlap of surface-labeled, internalized β1 integrin signal (blue) with AQP2 (red) and GFP-clathrin (green, upper panel) as well as GFP-rab11 (green, lower panel) is observed in vesicles. Scale bar, 5 μm in A; 2 μm in C.
accumulation of integrin β1, and by extension, probably also in AQP2 knockout animals.

**AQP2 Promotes Cell Migration by Facilitating the Turnover of Integrin β1 in the FAs**

The internalization and/or recycling of integrins affects formation and turnover of FAs as mentioned previously. We hypothesized that persistent surface retention of integrin β1 could delay the recycling/turnover of FAs and, therefore, inhibit cell migration. We applied TIRF microscopy to monitor the dynamic trafficking of integrin β1 in FAs in MDCK cells transfected with a GFP-integrin β1 chimera. This GFP-integrin chimera colocalized reliably with FA marker vinculin (Supplemental Figure 7). Our data showed that expression of WT-AQP2 accelerated the linear migration of integrin β1 signals in FAs concomitant with rapid cell movement on a fibronectin-coated surface (Supplemental Movies 4–6). In contrast, in untransfected cells, and especially in cells expressing the AQP2 RGD/A mutant, we observed more stable integrin β1 signals in stable FAs and much less cell movement over 30 minutes (Figure 6A). We quantified a significantly higher percentage of motile versus stationary FAs in MDCK-AQP2 cells than MDCK AQP2-RGD/A cells and untransfected cells (Figure 6B). Therefore, AQP2 promotes cell migration by facilitating mobilization/turnover of integrin β1 in the FAs. Furthermore, this effect of AQP2 on integrin β1 turnover in the FAs was only observed in cells grown on fibronectin−, but not on collagen-coated surfaces (Supplemental Figure 8), suggesting the requirement of specific integrin-ECM interaction and/or signaling.

**DISCUSSION**

We show here that AQP2 interacts with integrins, especially integrin β1 via its RGD motif. By interacting with integrin β1, AQP2 modulates integrin trafficking/surface presentation and turnover at the FAs, thereby contributing to cell motility and epithelial morphogenesis in vitro, and possibly playing an important role in the development and maintenance of kidney tubular structure and function in animals as well.

**Interaction of AQP2 and Integrins**

Our data suggest an interaction of AQP2 and integrin β1 through an RGD motif of AQP2. Integrins recognize ligands via an RGD motif, an essential adhesive sequence present in proteins such as fibronectin. Although, it has been shown that the β subunit (e.g., β3) can directly interact with certain ligands, and multiple discontinuous RGD recognizing sites have been identified, such as Asp109-Glu171 and a DXSXS motif that is highly conserved in all integrin β subunits. It is commonly accepted that both integrin α and β subunits are required for forming a ligand recognition and binding interface for RGD containing ligands. We have detected by immunoprecipitation the interaction of AQP2 with integrin subunits β1 and α5. As for most other integrin-ligand interactions, the interaction of AQP2 and integrin β1 probably requires the co-presence of the α subunit to form a coordinated interface for ligand recognition, specification and interaction. Supporting this hypothesis, we have detected the association of AQP2 with an RGD binding integrin α5β1, but not integrin α2β1, a non-RGD binding integrin. However, whether AQP2 can interact with a dimerized receptor or single subunit on the surface or inside the cells during their trafficking is a complex question and requires further characterization in future studies.

The ECM plays a critical role in determining or modifying integrin signaling and a variety of integrin-associated functions.
Despite the identification of integrins as adhesion molecules that mediate cell and ECM interaction, over the past several years, there has been a growing recognition of the importance of integrin-ion channel interaction in cell biology. Integrins form macromolecular complexes with ion channels and regulate ion transport across the plasma membrane, thereby contributing to cellular processes, such as motility, differentiation, and proliferation mediated by integrins.\(^{46,47}\) It has recently been reported that through interaction with the RGD motif of AQP2, integrin modulates AQP2 trafficking via cAMP and calcium signaling inside the cell.\(^{18}\) On the other hand, our report shows that by interacting with integrins, a non-ECM protein, AQP2 is involved in integrin-mediated cell migration and epithelial morphogenesis, which is an unusual function for this classic water channel. Moreover, our preliminary observations suggest a differential effect of ECM components on the migratory effect of AQP2. However, how the ECM modulates integrin-AQP2 interaction, AQP2 trafficking, and AQP2-mediated integrin turnover is a complex subject that needs to be elucidated with further mechanistic studies.

**Water Permeability and the Promigratory Effect of AQP2**

The presence of active water transport concomitant with dynamic ion movement at the leading edge in migrating cells has been proposed but not definitively proven due to inability to directly measure water flux in leading edges. Nevertheless, some other aquaporins have been reported to be involved in cell motility, especially AQP1, which has been implicated in migration of endothelial cells and renal epithelial cells.\(^{48,49}\) This effect was attributed to its water channel function in regions of cell extension. However, AQP1 null mice have no significant kidney structural defects, in contrast to dramatic abnormalities in AQP2 null mice.\(^{10,50}\) Although we cannot rule out completely that water-transporting activity mediated by AQP2 plays a role in cell migration, our data show that both wild-type AQP2 and AQP2 RGD/A responded to vasopressin stimulation by increasing membrane accumulation of AQP2 in LLCPK1 cells. No significant difference of water permeability was detected between the wild-type AQP2 and AQP2 RGD/A-expressing MDCK cells with or without forskolin stimulation. Therefore, we believe that water permeability per se is unlikely to play a significant role in the migration-inducing property of AQP2 in epithelial cells, and/or its effects on renal tubular structure and function in our tested systems.

**AQP2 Modulates Internalization/Trafficking of Integrin β1**

As mentioned above, similarly to AQP2, integrins are subjected to highly dynamic endocytosis and constitutive recycling.\(^{51,52}\) Disruption of integrin trafficking impairs cell spreading and migration. We postulate that through physical interaction, AQP2 affects the trafficking and subcellular distribution of integrin β1. Previous investigation of the trafficking of AQP2 and integrins in separate studies revealed some common trafficking machineries shared by both molecules.\(^{5,41,52,53}\) Recent proteomic analysis has revealed the presence of integrin β1 in AQP2-containing vesicles from renal inner medullary collecting duct cells.\(^{40}\) Our current data further confirm the co-presence of integrin β1 and AQP2 in intracellular vesicles after integrin internalization, providing a physical basis for the hypothesis. Indeed, we found increased surface accumulation of integrin β1 in AQP2-null cells and more dramatically, in AQP2 RGD/A-expressing cells compared with cells expressing wild-type AQP2, which is consistent with the observation of increased basolateral retention of integrin β1 in AQP2 null animals. Additional trafficking studies using an endocytosis assay reveal that AQP2 promotes, whereas lack of AQP2 or, in particular, expression of AQP2 RGD/A inhibits the endocytosis of integrin β1. This is further translated into the extent of turnover of integrin β1 in FAs, and regulation of the rate of cell migration *in vitro*, and we propose that this may translate in
vivo as well. We cannot, however, exclude other possible mechanisms that may explain the neonatal mortality and tubular abnormalities in AQP2 null animals, for example, modulating the actin cytoskeleton network or regulating other cell signaling pathways via AQP2.

We demonstrated here that AQP2 is important for cell migration and integrin internalization in cell assays and using AQP2 knockout mice. However, we also consistently observed an apparent, dominant negative effect of the AQP2 RGD/A mutant in both MDCK (expressing a low level but functionally significant endogenous AQP2) and LLC-PK1 cells (which may not express endogenous AQP2) during cell migration (Figure 3C), basolateral polarization of integrin in polarized cells (Figure 5A), integrin endocytosis (Figures 4, A and B and 5, B and C) and integrin turnover in FAs (Figure 6). Although the exact mechanism of the dominant negative effect of AQP2 RGD/A is not yet known, we do not believe that it is due to an off-target effect. First, the AQP2 RGD/A contains a point mutation with predicted minimal alteration of protein sequence, structure, and conformation. Second, the expressed protein traffics properly in response to vasopressin and increases water permeability in response to forskolin, similar to wild-type AQP2 (Supplemental Figure 5), suggesting that there are no significant conformational and functional alterations of this AQP2 RGD/A mutant. We and others have shown that AQP2 interacts directly or indirectly with a broad spectrum of proteins involved in general trafficking pathways, including heat shock protein70, clathrin, dynamin, and the actin cytoskeleton.6–8 It is reasonable, therefore, to invoke a possible broader modulating role of AQP2 in vesicle trafficking through direct or indirect interaction of its cytoplasmic domain with components of the trafficking machinery, in addition to its interaction with integrin β1. Therefore, expression of wild-type AQP2 probably facilitates the trafficking of integrin β1 through more effective coupling of integrin β1 with the trafficking machinery compared with AQP2 null cells. AQP2 RGD/A could still associate with the protein complexes that are required for integrin trafficking, but because it cannot interact with integrins, it could inhibit normal integrin trafficking by competing for these proteins. This dominant negative effect of mutated proteins in regulating protein trafficking has been reported for cystic fibrosis transmembrane conductance regulator in many cases of cystic fibrosis, for example.54,55 Whether the AQP2 RGD motif is critical for tubule formation in vivo is not yet known and will be further investigated using AQP2 RGD/A knock-in animals when they become available. Nevertheless, we have provided here a unique example in which a seemingly unrelated membrane protein, AQP2, modulates the trafficking of an adhesion molecule, integrin β1, and contributes critically to basic cell biologic processes including cell migration. This paradigm could be applicable to other “unconventional” molecular interactions and other biologic systems, thus furthering our understanding of kidney epithelial biology and the pathophysiology of kidney diseases.

AQP2 Expression and Tubular Defects in Polyuric Animals

On the basis of our data, we hypothesize that lack of functional AQP2 may contribute to the tubular abnormalities seen in AQP2 knockout and transgenic animals. As mentioned above, the tubular damage could potentially be due to a secondary effect of massive polyuria, which overwhelms the tubular system causing “functional obstruction.” This could result in principal cell damage and reduced expression of AQP2, rather than being a primary defect of AQP2 membrane expression. Although this is a reasonable hypothesis, it is unlikely to be the major cause of tubular defects seen in animals at very early postnatal stages whose urinary concentrating capacity is not fully developed. Furthermore, other animals with similar level of polyuria, such as Brattleboro rats15 and NDI mice with increased expression of cAMP phosphodiesterase,56 do not necessarily have tubular defects.

Could AQP2 be a unifying factor that contributes to the tubular damage in all transgenic animals presenting with polyuria? Interestingly, after a literature search of multiple published polyuric animal models, we found that despite the modification or knockout of different target genes, the majority of transgenic animals that have concentrating defects with structural abnormalities also have drastically reduced AQP2 levels; genetically modified mice that have no reported structural abnormalities usually have normal AQP2 levels (Supplemental Table 1). However, there are at least two exceptions in which reduced expression of AQP2 is seen with an apparent absence of tubular damage: cirrhosis produced by bile duct ligation57,58 and hypercalcemia-associated polyuria.59 There are also animals that show significant tubular abnormalities with preserved expression of AQP2.60 Although our current data and observations in several polyuric mouse models support the link between reduced AQP2 and renal tubular abnormalities, other factors are also clearly involved. There is a growing volume of literature regarding the role of a wide range of channels and transporters in cell migration,61 some of which could also play important roles in the development of various segments of the renal tubule.

In conclusion, our data show that the water channel, AQP2, plays an important and novel role in regulating epithelial cell migration through interacting with integrin β1 and modulating its trafficking. This effect, mediated through FAs, could explain the severely compromised kidney morphology and function, leading to early mortality, seen in AQP2 knockout mice.

CONCISE METHODS

Generation of AQP2 Constructs, AQP2 Peptides, and Stable Cell Lines

Mutation of rat AQP2 second external loop RGD motif, AQP2 RGD/A was generated by site-directed mutagenesis (forward primer, CCA GTA GAA ATC CGT GGG GCA CTG GCT GTC ATG; and reverse
primer, CAT TGA CAG CCA GTG CCC CAC GGA TTT CTA CTG G), and was cloned into pcDNA1 vector. pcDNA1-rat AQP2 and pcDNA1-rat AQP2 RGD/A constructs were transfected into MDCK and LLCPK1 cells and individual stable cell lines were established as previously reported. AQP2 peptides spanning the second extracellular loop of AQP2 were synthesized by the Massachusetts General Hospital peptide/protein core facility, including AQP2 (EIRGDALVNALHNATA-lys-biotin) and AQP2-RGD/A (EIRGALAVNALHNATA-lys-biotin).

Wound Healing Assays
The glass bottom of a SmartSlide-6 Micro-Incubator (WaferGen Biosystems Inc) was coated with 5 µg/ml fibronectin or collagen before use. Stably transfected and untransfected MDCK or LLCPK1 cells were grown on the microincubator to confluence in DMEM containing 0.3% FBS. A scratch wound was created using a 200-µl pipette tip. The wounded monolayer was washed to remove nonadherent cells. Cell migration toward the wounded space was monitored using a Nikon Eclipse TE300 inverted microscope equipped with temperature controller and CO2 supplier. Images were taken every 15 minutes for 15 hours. Wound healing was quantified as the average linear speed of the moving wound edge over 15 hours using NIH ImageJ software.

Tubulogenesis Assays
We suspended 1×10⁶ cells/ml from a single cell suspension of stably transfected and untransfected LLCPK1 cells in 80% type I (BD Biosciences) collagen gel or 60% collagen and 20% Matrigel (BD Biosciences) mixture. After solidification of the cell/gel suspension, fresh DMEM with 10% FBS was added onto the cell/gel layer and incubated for 3–7 days to allow tubules to form. Phase contrast images were taken using a Hamamatsu digital camera attached to a Nikon Eclipse TE300 inverted microscope. Ten randomly selected 4× low-power images were taken for each gel culture. The number of tubular structures formed (defined as >1 branch), cell aggregation, and cyst formation were quantified.

Measurement of Osmotic Water Permeability
Water permeability assays were performed as described previously with modification. Briefly, stably transfected and untransfected MDCK cells preloaded with 20 µM calcein acetoxymethyl ester (Invitrogen) were incubated for 15 minutes in the presence or absence of forskolin (10 µM) at 37°C in a perfusion chamber. The rate of change of calcein fluorescence intensity inside cell was monitored using TIRF microscopy after first exposing cells to hypertonic (500 mOsm) and then switched to hypotonic (150 mOsm) perfusate.

Coimmunoprecipitation
Coimmunoprecipitation was performed as previously described. Briefly, stably transfected and untransfected LLCPK1 cells from a 100-mm tissue culture dish were harvested with trypsin/EDTA. Then, cells were lysed for 1 hour at 4°C in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 1 mM MgCl₂, 0.2 mM CaCl₂, and protease inhibitor cocktail. After clarification of the cell lysates by centrifugation at 12,000 g for 10 minutes at 4°C, immunoprecipitation was performed by incubation with the selected antibody for 2 hours at 4°C. The antigen antibody complexes were bound to protein A sepharose beads overnight at 4°C. The beads were washed three times with RIPA buffer before loading onto gel.

Cell Surface Biotinylation Assays
Transfected and untransfected stable MDCK cells were surface labeled with biotin using a modification of a previously described method. Adherent cells from 10-cm dishes were harvested with trypsin/EDTA, washed twice with PBS, and incubated with 1 mg/ml biotin (NHS-LC-Biotin, Pierce) in PBS. After a 30-minute incubation at 4°C with constant agitation, the reaction was quenched by Tris-buffered saline. Then cells were washed three times in PBS and pellets of cells were subjected to lysis and immunoprecipitation with streptavidin beads (Dynabeads M-280; Invitrogen) as described above. Proteins attached to the beads were resuspended in 30 µl of sample buffer and separated by 4%–12% SDS-PAGE.

Endocytosis Assays
Integrin internalization assays were performed as previously described. Briefly, untransfected and transfected stable MDCK cells were grown on glass coverslips overnight, pretreated with cycloheximide (50 µg/ml), and serum-starved in DMEM with 0.01% FBS. Monoclonal anti-integrin β1 antibody Ts2/16 was incubated with cells at 4°C for 1 hour. After washing to remove excess antibody, cell surface-labeled integrin β1 was allowed to internalize after incubation at 37°C. Cells were harvested at various time points and stained with Cy3 conjugated secondary antibody. Phalloidin-fluorescein staining was used to mark cell membrane boundaries. The fluorescence intensity of integrin β1 signal on cell membranes and inside cells throughout the time course of endocytosis was measured and analyzed using ImageJ software.

TIRF Microscopy
MDCK, MDCK-AQP2, and MDCK RGD/A cells were transiently transfected with a GFP-β1 integrin chimera for 48 hours. Cells were then seeded on precoated glass-bottomed dishes (fibronectin or collagen, 5 µg/ml) overnight. Single cell was observed using a 60× NA 1.45 apochromat objective on a Nikon TE2000-U microscope equipped with a TIRF illuminator and fiber optic–coupled laser illumination. For live cell imaging of GFP-β1 integrin, images were acquired every 30 seconds for a total of 30 minutes. The images were analyzed using NIH ImageJ software. Sixty TIRF images acquired every 30 seconds over 30 minutes were combined into an image stack and color coded by time. FA movement was observed as a rainbow-colored streak, whereas nonmoving FAs appeared as white colored puncta. To quantify the movement of FAs, the length of the rainbow-colored regions, representing the distance of movement, was measured. To quantify the percentage of moving FAs per cell, the number of moving FAs was compared with all of the FAs that were visible at time zero in individual cells.

Statistical Analyses
Statistical analyses were performed with GraphPad Prism software (La Jolla, CA). All groups were initially compared via one-way
ANOVA. A two-tailed t test was applied to determine a difference between individual groups and a P value <0.05 was considered significant. Data were expressed as mean ± SEM. Error bars indicated SEM in each histogram. Data were obtained from at least three independent experiments in each experimental condition.

ACKNOWLEDGMENTS

We thank Dr. Martin Hemler (Harvard Medical School) for providing integrin α2 antibody, and Dr. Tom Walz (Harvard Medical School) for providing the GFP-AQP0 plasmid.

H.I.L. is supported by an NIH KO8 grant (DK075940) and a Gottschalk research grant from the American Society of Nephrology (ASN). D.B. is supported by a NIH grant (DK38452). W.R. is supported by a NIH training grant (NIH 5 T32 DK007540–24). The Microscopy Core Facility of the Program in Membrane Biology receives additional support from the Boston Area Diabetes and Endocrinology Research Center (NIH DK-57521) and from the Center for the Study of Inflammatory Bowel Disease (NIH DK-43351).

DISCLOSURES

None.

REFERENCES

13. Agre P, Kozono D: Aquaporin water channels: Molecular mechanisms for providing the GFP-AQP0 plasmid.

BASIC RESEARCH


