Integrin Signaling Modulates AQP2 Trafficking via Arg-Gly-Asp (RGD) Motif

Grazia Tamma¹, Domenica Lasorsa¹, Marianna Ranieri¹, Lisa Mastrofrancesco¹, Giovanna Valenti¹ and Maria Svelto^{1,2}

¹Department of General and Environmental Physiology, University of Bari, Bari, ²Centro di Eccellenza di Genomica in campo Biomedico ed Agrario (CEGBA)

Key Words

Aquaporins • Integrin • Trafficking

Abstract

Aquaporin-2 (AQP2) increases the water permeability of renal collecting ducts in response to vasopressin. Vasopressin stimulation is accompanied by a profound remodeling of actin cytoskeleton whose dynamics are regulated by crosstalk between intracellular and extracellular signals. Here, we report that AQP2 contains a conserved RGD domain in its external C-loop. Co-immunoprecipitation experiments demonstrated that AQP2 binds integrin $\beta 1$ in renal tissue and in MCD4 cells. To investigate the role of this interaction on AQP2 trafficking, cells were exposed to synthetic RGD-containing peptides, GRGDNP or GRGDSP, able to bind certain integrins. Incubation with these peptides increased the membrane expression of AQP2 in the absence of hormonal stimulation as assessed by confocal analysis and cell surface biotinylation. To identify the signals underlying the effects of peptides on AQP2 trafficking, some possible intracellular messengers were evaluated. Exposure of MCD4 cells to GRGDNP increased

intracellular cAMP as assessed by FRET studies while GRGDSP increased intracellular calcium concentration. Taken together, these data propose integrins as new players controlling the cellular localization of AQP2, via two distinct signal transduction pathways dependent on cAMP and calcium respectively.

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Introduction

Renal collecting duct water permeability is regulated by the hormone vasopressin which controls the expression [1] and cellular distribution of the water channel AQP2 [2-4]. Vasopressin binds its cognate receptor V2R stimulating a cAMP-dependent signal transduction cascade leading to the activation of protein kinase A (PKA) which phosphorylates AQP2 at serine 256 [5-7]. This event is essential to promote the translocation of AQP2-bearing vesicles to the apical plasma membrane, thus ensuring collecting duct water reabsorption [5].

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Dr. Grazia Tamma
Department of General and Environmental Physiology
University of Bari, Via Amendola 165/A, 70125 Bari (Italy)
Tel. +39 080 5443414, Fax+39 080 5443388
E-Mail g.tamma@biologia.uniba.it

Although membrane abundance of AQP2 is mainly under vasopressin control, it has also been shown that the cell surface expression of AQP2 might be tightly modulated by the synergic activity of various small molecules and hormones such as PGE2 [8, 9], bradykinin [10], dopamine, ATP [11] and ANP [12]. Importantly, signal pathways controlled by these agonists are often associated with a profound reorganization of the actin cytoskeleton. Relaxation of actomyosin has been proposed to play a role in facilitating the trafficking of intracellular vesicles [13], though it is well established that actin reorganization is an important event occurring during exocytosis. This is particularly true for AQP2 whose cellular distribution is regulated by phosphorylation and interaction with the actin cytoskeleton [14, 15]. Several intracellular pathways, involving Rho signaling, participate in the complex regulation of actin filament stability. PKA phosphorylates RhoA at serine 188 and this phosphorylation increases the interaction with GDP dissociation inhibitor (GDI), inhibiting binding with downstream targets independently of the GTP/GDP state [8, 16]. Therefore, cAMP signal transduction pathway mediates down-regulation of RhoA, causing a partial depolymerization of actin filaments, thus facilitating the translocation of AQP2 vesicles from an intracellular pool to the apical plasma membrane [8]. It is well accepted, however, that Rho activity depends on integrins [17, 18] which can be considered *cell-hubs* integrating extracellular and intracellular signals. Integrins are transmembrane $\alpha\beta$ heterodimers binding specific ligands and having distinct structures. 18 α and 8 β isoforms have been isolated in humans forming 24 heterodimers. On ligand binding, integrins transfer extracellular signals into the cell but intracellular signals can also modulate integrin affinity to certain ligands. In addition to modulating cell attachment, integrin-dependent cell binding to multi-adhesive extracellular matrix (ECM) molecules might initiate several cellular responses, including changes in gene expression, intracellular ion concentration, the activation and generation of traditional second messengers [19, 20]. The Arg-Gly-Asp (RGD) sequence is a known integrin-binding motif, originally identified in fibronectin [21] and subsequently found in several other matrix proteins and ion channels [22]. Indeed, integrins are transmembrane transducers between external molecules and the actin cytoskeleton. Several proteins on the intracellular side, such as the talin, vinculin and ERM (ezrin, radixin and moesin) actin binding proteins, bridge the intracellular domain of integrin to the cytoskeleton building complex and local intracellular interactoma [23]. This constitutes the molecular base for

integrin to participate in many cellular processes such as adhesion, proliferation, shape, polarity, trafficking and differentiation. The recent development of integrin-deficient mice shed more light on the role of these proteins on kidney functions. Knockout mice for integrin $\alpha 2$, $\alpha 3$ and $\alpha 8$ have defects in renal morphogenesis and enhanced glomerular and tubulointerstitial matrix deposition [24-27]. Mice deficient for $\beta 1$ integrin in the renal collecting duct develop progressive renal dysfunctions, including dilated tubules, cyst formation and polyuria. Consistent with these observations, real time PCR of laser captured renal medulla, isolated from mutant and wild type mice, shows a significant decrease in V2R and AQP2 in knockout mice, providing a possible explanation for the observed polyuric phenotype [28]. On the other hand, AQP2-deficient mice have severe hydronephrosis, obstructive nephropathy and tubular dysfunctions [29]. Furthermore, proteomic analysis revealed that immunoisolated AQP2 vesicles contain integrin $\alpha 2$, $\alpha 3$, $\alpha 5$ and $\beta 1$ [30] suggesting a possible involvement of integrin signaling in AQP2 trafficking. We show here that AQP2 contains the conserved RGD domain in its external C-loop and interacts with integrin $\beta 1$. Treatment with synthetic RGD-containing peptides affects the cellular distribution of AQP2, activating two distinct signal-transduction pathways dependent on cAMP and calcium, respectively.

Materials and Methods

Chemicals and reagents

To detect the total amount of AQP2, we raised antibodies against 20 amino acids located just N-terminal from the poly-phosphorylated region of rat AQP2 (CLKGLEPDTDWEEREVRRRQ). Integrin beta-1 was purchased from Santa Cruz Biotechnology (DBA, Milan, Italy). Integrin peptides (GRGDSP, GRGDNP) were from Anaspec (Eurogentec Group, Fremont CA, USA). Protein A-sepharose was from Sigma (Milan, Italy). Biocytin Hydrazide and streptavidin beads were purchased from EZ-Link[®] Pierce. Fura-2AM, and Alexa 488 antibodies were purchased from Molecular Probes, Eugene, OR, USA.

Cell culture and treatments

Mouse M1-hAQP2 (MCD4) cells, stably transfected with human AQP2 [31, 32], were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37°C in 5% CO₂. MCD4 cells were seeded at 3x10⁵ cells/cm² and grown to confluence for 3 days. After overnight treatment with indomethacin (5x10⁻⁵ M), cells were left under basal condition or stimulated with forskolin (10⁻⁵ M for 45 min). Alternatively, cells were incubated with integrin peptides (10 μ M), in the absence or in the presence of forskolin (10⁻⁵ M

for 45 min). As reported [32, 33], addition of the prostaglandin synthesis inhibitor indomethacin was needed to reduce basal cAMP and was present with all treatments.

Immunofluorescence

Immunocytochemistry was performed as described [34]. Briefly, MCD4 cells were grown, treated as described above, and fixed for 30 min with 4% paraformaldehyde in PBS. Following quenching of aldehyde groups with 50mM NH_4Cl in PBS for 15 min, samples were permeabilized with 0.1% TritonX-100 in PBS for 5 min, blocked with 1% PBS-BSA for 30 min and incubated for 2 hours with a 1:1000 dilution of AQP2 antibodies. After washing three times with PBS-BSA, samples were incubated with 1:1000 diluted goat anti-rabbit antibodies coupled to Alexa-488 in PBS-BSA for 1 hour. Next, cells were rinsed three times with PBS and mounted on glass slides with Mowiol. Images were obtained with a Leica TCS SP2 (Leica Microsystems, Heerbrugg, Switzerland).

Immunoprecipitation

Immunoprecipitation experiments were performed as previously described [6, 35]. Briefly, MCD4 cells or total kidney were homogenized and lysed with 1% Triton X-100, 150mM NaCl, 25mM Hepes (pH 7.4) in the presence of protease inhibitors (1mM PMSF, 2 mg/ml leupeptin and 2mg/ml pepstatin A). The supernatants were precleared with 50 μl of immobilized protein-A and incubated O/N with anti-AQP2 antibodies coupled to protein A-sepharose. As negative control, lysates were incubated with non-specific rabbit IgG. The immunocomplexes were washed three times, resuspended in 50 μl of Laemmli buffer and subjected to immunoblotting using integrin beta-1 and AQP2 antibodies.

Cell surface biotinylation

Biotinylation was carried out according to the protocols provided by the reagent manufacturer (PIERCE) with some adaptations. MCD4 cells were cultured on 6-well filters in DMEM for 4 days and treated as described above. Cells were washed thoroughly with ice cold Coupling Buffer (NaPO_3 0.1 M, NaCl 0.15 M at pH 7.2) before being subjected to oxidation with Na-meta-periodate 20 mM in Coupling Buffer for 30 minutes on ice in the dark. Following three rounds of washing with Coupling Buffer, cell surface glycoproteins were labeled with 5mM Biocytin Hydrazide (EZ-Link[®] Pierce) for 30 minutes. The biotinylation buffer was removed, cells were incubated with Quenching Solution (NH_4Cl 50 mM in PBS at pH 7.2) for 5 minutes and washed three times with Coupling Buffer. Cells were solubilized with lysis buffer (Triton-X 100 1%, SDS 0.01% in PBS), and supplemented with 2 $\mu\text{g}/\text{ml}$ Pepstatin, 2 $\mu\text{g}/\text{ml}$ Leupeptin and 2mM PMSF for 30 minutes. The lysates were homogenized using ultrasonic homogenizer at 40 Hz for 20 seconds and centrifuged at 12,000 $\times g$ for 20 minutes. An aliquot of the supernatants (20 μl) was diluted in Laemmli buffer while the remaining biotinylated proteins were pulled down with immobilized streptavidin beads. The complexes were washed 3 times with wash Buffer (Triton-X 100 0.5 %, SDS 0.01 % in PBS), denatured in Laemmli buffer for 30 minutes at 37 °C and analyzed by SDS-PAGE and immunoblotted.

Immunoblotting and densitometric analysis.

SDS-PAGE and blotting, blocking, antibody incubation, and chemiluminescence of the membranes were performed as previously described [8]. AQP2 and integrin beta-1 antibodies were used at 1:1000 and 1:500 dilution respectively. As secondary antibodies goat anti-rabbit (AQP2) or goat anti-mouse (beta-1) HRP-coupled secondary antibodies were used. Representative figures are shown.

Statistical analysis

One-way ANOVA followed by Newman-Keuls Multiple comparison test were used for the statistical analysis. All values are expressed as mean $\pm$ SEM. A difference of $P < 0.05$ was considered statistically significant.

Video Imaging Measurements

In fluorescence measurements, the coverslips with dye-loaded cells were mounted in a perfusion chamber (FCS2 Closed Chamber System, BIOPTECHS, Butler, U.S.A.) and measurements were performed using an inverted microscope (Nikon Eclipse TE2000-S microscope) equipped for single cell fluorescence measurements and imaging analysis. The sample was illuminated through a 40X oil immersion objective (NA = 1.30).

Intracellular calcium measurements

MCD4 cells were loaded with 10 μM Fura2-AM for 15 min at 37°C in DMEM. Ringer's Solution used to perfuse cells during the experiments contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM Hepes, 5 mM Glucose, 1 mM CaCl_2 , pH 7.4. The fura-2 loaded sample was excited at 340 and 380 nm. Emitted fluorescence was passed through a dichroic mirror, filtered at 510 nm (Omega Optical, Brattleboro, VT, USA) and captured by a cooled CCD camera (CoolSNAP HQ, Photometrics). Fluorescence measurements were carried out using Metafluor software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada). Data are reported as mean values $\pm$ SEM with n equal to the number of cells. Response were analyzed as changes in fluorescence intensity and compared with changes induced by ATP as control stimulus. The significance of the observations was evaluated by Student's t test for paired data, with $P < 0.05$ considered to be statistically different.

Cell transfection

MCD4 cells were seeded a day prior to transfection at 80% confluence. 25 μl of PEI (1 $\mu\text{g}/\mu\text{l}$) was dissolved with 25 μl of NaCl (150mM) and incubated for 5 min at room temperature. In parallel, 2 μg of DNA was diluted in NaCl 150mM to a final volume of 50 μl . After 5 min, solutions were mixed, vortexed and incubated for 20 min at room temperature. Transfecting solution was then added to 2 ml of complete medium and incubated for 8 hours. FRET experiments were performed 48 hours after transfection.

Fluorescence Resonance Energy Transfer (FRET) measurements

FRET experiments were performed as described [36, 37]. Briefly, MCD4 cells were transiently co-transfected with

<i>Extracellular C-loop of AQP2</i>	
Species	Sequence
Human	TPADI RGD LAVNALSNSTTA
Mouse	TPVEI RGD LAVNALHNNATA
Rat	TPVEI RGD LAVNALHNNATA
Bovine	TPPAI RGD LAVNALNNNSTA
<i>Extracellular C-loop of AQP3</i>	
Species	Sequence
Human	ADNQLFVSGPNGTAGIFATYPSGHLDM
Mouse	ANNELFVSGPNGTAGIFATYPSGHLDM
Rat	AGNELVSGPNGTAGIFATYPSGHLDM
Bovine	ANNQLIVSGPNGTAGIFATYPSGHLDM
<i>Extracellular C-loop of AQP4</i>	
Species	Sequence
Human	TPPSVVGGLGVTMVHGNLT
Mouse	TPPSVVGGLGVTTVHGNLT
Rat	TPPSVVGGLGVTTVHGNLT
Bovine	TPPSVVGGLGVTTVHGNLS
<i>Extracellular C-loop of AQP6</i>	
Species	Sequence
Human	MPGDIRETLGINVVRNSVST
Mouse	TPGGIRETLGVNVVHNSTST
Rat	TPGGVRETLGVNVVHNSTST

Table 1. Alignment analysis of collecting duct aquaporin sequences in different species: the RGD motif is highly conserved in AQP2 among species.

plasmids encoding the regulatory and the catalytic subunit of PKA fused to cyan fluorescent protein (RII-ECFP) and yellow fluorescent protein (C-EYFP) respectively (1 μ g per each plasmid) using the PEI procedure. RII-ECFP and C-EYFP have been previously described [38] and were kindly provided by Prof. Zaccolo. FRET measurements were carried out using MetaMorph software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada). ECFP and EYFP were excited at 430 or 512 nm, respectively; fluorescence emitted from ECFP and EYFP was measured at 480/30 and 545/35 nm, respectively. FRET from ECFP to EYFP was determined by excitation of ECFP and measurement of fluorescence emitted from EYFP. Corrected nFRET values were determined according to Ritter [39].

Results

AQP2 binds integrin β 1

Sequence analysis of renal collecting duct aquaporins revealed that AQP2 contains the minimal integrin recognition sequence RGD in its extracellular C-loop. Alignments demonstrated that the RGD motif is highly conserved in AQP2 among species (Table 1).

Because the presence of the conserved RGD binding sequence does not ensure a real protein-protein interaction between integrin β 1 and its cognate proteins, co-immunoprecipitation experiments were performed,

Fig. 1. Co-immunoprecipitation and Western blotting studies. Kidney and MCD4 cells were lysed and subjected to immunoprecipitation (IP) using AQP2 antibodies. Immunocomplexes were revealed with β 1-integrin (expected weight 138kDa) and AQP2 (expected weight 29kDa) antibodies. As negative control, kidney or cell lysates were incubated either with protein A-Sepharose, in the absence of specific AQP2 IgG, or with non-specific IgG. Lysates were loaded and probed with both antibodies.

Fig. 2. Confocal microscopy analysis of AQP2 immunolocalization in MCD4 cells. Cells were grown on permeable support to complete confluence, fixed and stained with antibodies against AQP2. In resting cells, AQP2 was mainly detectable in sub-apical vesicles (-FK). After FK treatment, AQP2 localized to the apical membrane (+FK). Treatment with GRGDNP (10 μ M) and GRGDSP (10 μ M) caused AQP2 redistribution towards the apical plasma membrane regardless forskolin stimulation.

using either renal tissue or MCD4 cells [31]. To this end, kidney and cell lysates were incubated with antibodies specific for AQP2. Immunocomplexes were precipitated with protein-A Sepharose and analyzed by immunoblotting with antibodies specific for integrin β 1; AQP2 antibodies were used as control. As negative control, kidney or cell lysates were incubated either with protein A-Sepharose, in the absence of specific AQP2 IgG, or with non-specific IgG. The results obtained (Fig. 1) indicate that AQP2 can specifically complex with integrin β 1 in renal tissue and in MCD4 cells.

Fig. 3. Biotinylation assay with Biocytin Hydrazide in MCD4 cells. (A) Immunoblotting analysis of total and apical AQP2 in control conditions and after treatment with Forskolin 10^{-5} M or GRGDNP (10 μ M) and GRGDSP (10 μ M). (B) Densitometric analysis of the 29 kDa biotinylated AQP2 band normalized to total AQP2 (means $\pm$ SE; * $P < 0.05$).

Synthetic RGD-containing peptides control the cellular localization of AQP2

RGD-containing peptides have been used as antagonists of integrin functions *in vivo* [40]. It is also true, however, that the ligand-binding ability of these peptides make them good agonists for activating integrin-dependent signaling cascades [41].

To evaluate the physiological consequences of the interaction between the water channel AQP2 and integrin β 1, MCD4 cells were incubated either with GRGDNP or with GRGDSP, integrin peptides, able to affect integrin signaling. Confocal analysis revealed that preincubation with both peptides increased the cell surface abundance of AQP2 regardless of forskolin stimulation (Fig. 2). As expected, under basal condition AQP2 located in vesicles whereas forskolin stimulation caused AQP2 translocation from an intracellular pool to the apical plasma membrane.

To verify whether the apical localization observed by confocal studies coincides with a real insertion of AQP2 into the membrane, cell surface biotinylation experiments were performed. To increase the yield of the detectable membrane-inserted AQP2, the cell surface was labeled with biocytin hydrazide which binds glycoproteins specifically, because conventional biotinylation reagents bind lysine residues, which are poorly exposed in AQP2, on the extracellular side [42]. Biotinylated proteins were purified from cell lysates with immobilized streptavidin. Compared to untreated cells

Fig. 4. Evaluation of PKA activity by FRET analysis. (A) Schematic model showing regulatory (RII) and catalytic (C) subunits of PKA fused with CFP and YFP used to monitor PKA activity. Low concentrations of cAMP are associated with a decrease in PKA activity, which results in a specific interaction between catalytic and regulatory subunits. When the cAMP level increases, regulatory subunits detach from the active catalytic subunits, impairing the fluorescence energy transfer process from the donor (CFP) to the acceptor (YFP). (B) Histogram (means $\pm$ SE; * P <0.05) compares changes of NFRET Ratio, between Forskolin, GRGDNP and GRGDSP versus control conditions.

Fig. 5. Intracellular Ca^{2+} measurements. (A) Effect of GRGDNP and (B) Effect of GRGDSP, on free cytosolic Ca^{2+} signaling of MCD4 intact cells loaded with Fura-2 AM. The right-hand side of each panel histogram (means $\pm$ SE; * P <0.001) shows changes expressed as Δ Fura-2 Ratio compared to the response to ATP used as control.

(CTR), forskolin stimulation (FK) significantly increased the abundance of AQP2 at the apical plasma membrane, as indicated by the increase in the amount of membrane AQP2 (M.AQP2) normalized with respect to total AQP2 (T.AQP2) (FK=3.87 $\pm$ 0.38 vs CTR=1 $\pm$ 0.27 N=3 * P <0.05) (densitometry on the right in Fig. 3). Similarly, incubation with integrin peptides, GRGDNP or GRGDSP, resulted in a significant increase in cell-surface-expressed AQP2 compared to untreated cells as indicated by the increase

in the ratio M.AQP2/T.AQP2 (GRGDNP=5.49 $\pm$ 1.19, GRGDSP=3.31 $\pm$ 0.36 vs CTR=1 $\pm$ 0.27 N=3 * P <0.05) (densitometry on the right in Fig. 3). Together, these observations indicate that both peptides caused redistribution of AQP2 towards the plasma membrane. As known, AQP2 localization is modulated by intracellular level of cAMP which activates PKA thus resulting in the translocation of AQP2 vesicles to the plasma membrane [5].

Synthetic RGD-containing peptides affect cAMP and intracellular calcium concentration

To investigate the molecular mechanisms underlying the effect of integrin peptides, the activity of PKA was monitored by FRET. The regulatory and catalytic subunits of PKA, fused with the respective green fluorescent protein mutants, were used for measuring changes in FRET that correlate with PKA activity and then with local changes in intracellular cAMP levels (Fig. 4A) [38]. MCD4 cells were co-transfected with RII-ECFP and Cat-EYFP and FRET signals were measured under treatments described above. Compared to untreated cells, NFRET signals decreased under incubation with GRGDNP, consistent with an activation of PKA. A similar decrease was detected under forskolin treatment (FK=0,6±0,06 vs CTR=1±0,12 *P<0,05) as positive control. In contrast, GRGDSP peptide did not alter the basal activity of PKA (GRGDSP=1,02±0,13 vs CTR=1,0±0,12) (Fig. 4B).

Therefore, these data might explain the mechanism of GRGDNP action on promoting the targeting of AQP2 at the plasma membrane, while the mechanism of GRGDSP action is not explained by this pathway. We then analyzed the possibility that GRGDSP activate AQP2 trafficking by mobilizing intracellular calcium. It has been demonstrated in fact that the ligand-mimetic peptide GRGDSP mobilizes the intracellular calcium in different cell models [43–45]. Fig. 5 shows the comparison between the effects on intracellular calcium concentration induced by both integrin peptides and those elicited by the classical calcium agonist ATP (100μM). While GRGDNP did not cause any alteration of intracellular calcium concentration compared to ATP as internal control (Fig. 5A, GRGDNP=0.035±0.017 vs ATP 1±0.11, N=73; *P<0.001), GRGDSP elicited a transient increase in calcium (Fig. 5B, GRGDSP 0.42±0.023 vs ATP 1±0.18 N=61, *P<0.001).

Discussion

In the present study, we propose integrins as new players regulating AQP2 trafficking in renal collecting duct principal cells. Quite interestingly, AQP2 is the only collecting-duct water channel having a conserved RGD integrin-binding domain at the external C-loop (Tab.1). A large number of studies have shown that RGD is a typical motif of cell adhesion molecules (CAMs) and many ECM proteins. Moreover, a physical interaction between ion channels and integrin β1, via RGD, has been demonstrated [17]. Numerous data showed an intricate interplay

between integrins, channels and cellular messengers. Particularly, integrins transmit bidirectional signals across the plasma membrane, participating in many cellular processes modulating the activity of several membrane proteins. In line, integrin β1 interaction with K⁺ GIRK channels (G protein-activated inward rectifier K⁺ Channels) plays a role in controlling the activity and membrane abundance of this channel [22].

In this study, we show that AQP2 binds integrin β1 in native renal tissue and in MCD4 cells (Fig. 1), suggesting a possible signaling relation between these proteins. To investigate the possible involvement of integrin signaling on AQP2 trafficking, we took advantage of integrin peptides containing the RGD motif which are known to affect “outside-in signaling”. It has been previously reported that peptides containing the RGD sequence bind integrin receptor, inhibiting cell adhesion by competing with the integrins/matrix interaction causing various cellular effects [46]. Notably, for many integrin ligands, the amino acid following the active RGD sequence is crucial for substrate selectivity. On the other hand, the binding specificity of integrins depends on residues outside the RGD domain. GRGDNP shows a higher ability to bind α5β1 than αvβ3, whereas GRGDSP interacts better with αvβ3 [47]. Increasing numbers of studies show that RGD-containing peptides exert numerous responses at intracellular level. In the present study, we found that both peptides, GRGDNP and GRGDSP, increased the abundance of AQP2 at the apical plasma membrane (Fig. 2–3) likely interpretable as demonstrating an ability of the peptides to target α5β1 and αvβ3 respectively. In contrast, as negative control, cyclic-RGD or RGE peptides do not exert any effect on AQP2 localization (data not shown). Whether GRGDNP and GRGDSP peptides actually interact with α5β1 and αvβ3, in our experimental model, remains to be established even though their ability to affect the localization of AQP2 has been shown here. As the next step, to address the intracellular mechanism underlying the effect of integrin peptides on AQP2 relocation, changes in the intracellular levels of traditional second messengers were evaluated. Consistent with the apical localization of AQP2, GRGDNP, similarly to stimulation with forskolin, activates cAMP-dependent signaling, as assessed by FRET studies (Fig. 4). GRGDSP elicited a significant increase in intracellular calcium levels (Fig. 5) without affecting PKA activity (Fig. 4). While it is clearly accepted that AQP2 translocation is associated with an increase in intracellular cAMP, the role of calcium in the translocation of AQP2 is still quite controversial. In some experimental models, vasopressin stimulation results

in a significant increase in both cAMP and Ca^{2+} [48, 49]. Quite interestingly, cAMP analogs are known to induce Ca^{2+} oscillation via another cAMP-effector, Epac (Exchange Protein directly activated by cAMP) without affecting the activity of PKA [50]. Epac is a known GEF for the small GTPase Rap1 which is involved in controlling integrin-dependent cell adhesion and cell-junction formation [51, 52]. Epac-specific cAMP agonist (8-pCPT-2'-O-Me-cAMP) mimics AVP-elicited Ca^{2+} mobilization, resulting in the translocation of AQP2 vesicles to the apical plasma membrane in a ryanodine-dependent manner [50]. In this respect, it has been reported that Ca^{2+} release, induced by the synthetic integrin peptide, GRGDSP, is ryanodine-sensitive and diffuses as Ca^{2+} waves through the entire cell [43, 53, 54]. Together, these observations raise the hypothesis that integrins, on the plasma membrane of renal principal cells, might be considered pivotal transducers regulating AQP2 trafficking by mobilizing intracellular calcium. In this context, however, the question of whether integrins can be considered upstream of Epac proteins remains to be investigated. Epac activation is associated with integrin $\beta 1$ -mediated adhesion to fibronectin [55] and reorganization of actin cytoskeleton possibly via downregulation of Rho proteins [56, 57]. Indeed, both integrin peptides, GRGDNP and GRGDSP, might converge their effect on Rho signaling which is implicated in the regulation of AQP2 trafficking [8, 58]. Consistently, GRGDNP treatment results in a significant increase in PKA activity which phosphorylates several intracellular targets, including RhoA at Ser188, resulting in its inhibition. Rho inhibition is in turn associated with actin reorganization resulting in an increase in the cell-surface expression of AQP2 [8]. Conversely,

GRGDSP, by increasing intracellular levels of Ca^{2+} , might play a role in controlling the activity of several cytoskeletal proteins known to modulate actin dynamics.

While a clear picture describing this complex mechanism regulating the cellular localization of AQP2, via the integrin signaling pathway, needs further investigation, our results demonstrate for the first time the involvement of integrins in AQP2 trafficking regulation. In particular, we show that: a) AQP2 is the only water channel containing the conserved integrin binding motif RGD; b) AQP2 interacts with integrin $\beta 1$; c) AQP2 localization is fine-tuned by integrin peptides via cAMP and Ca^{2+} signaling, respectively.

Of note, many recent studies have shown that RGD peptides are powerful tools for the treatment of several diseases. The signaling pathway regulating AQP2 cellular distribution may be considered a suitable target for the therapeutical treatment of diseases associated with body water imbalance. In this scenario, novel custom-designed RGD-containing peptides, targeting AQP2 specifically, might be considered for improving the renal concentrating defects associated with a dysregulation of the V2R-AQP2 axis.

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