Role of NHERF1, Cystic Fibrosis Transmembrane Conductance Regulator, and cAMP in the Regulation of Aquaporin 9*

Christine Pietrement1, Nicolas Da Silva5, Claudia Silberstein1, Marianne James1, Mireille Marsolais9, Alfred Van Hoek†‡, Dennis Brown§, Nuria Pastor-Soler**, Nadia Ameen†, Raynald Laprade†, Vijaya Ramesh55, and Sylvie Breton*††

From the 1Center for Systems Biology, Program in Membrane Biology/Nephrology Division and the 2Center for Human Genetic Research, Massachusetts General Hospital, Boston, Massachusetts 02114, the 3Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215, the 4Groupe d’Étude des Protéines Membranaires, Université de Montréal, Montréal, Canada, and the **Renal-Electrolyte Division and ††Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Water and solute transport across the plasma membrane of cells is a crucial biological function that is mediated mainly by aquaporins and aquaglyceroporins. The regulation of these membrane proteins is still incompletely understood. Using the male reproductive tract as a model system in which water and glycerol transport are critical for the establishment of fertility, we now report a novel pathway for the regulation of aquaporin 9 (AQP9) permeability. AQP9 is the major aquaglyceroporin of the epididymis, liver, and peripheral leukocytes, and its COOH-terminal portion contains a putative PDZ binding motif (SVIM). Here we show that NHERF1, cystic fibrosis transmembrane conductance regulator (CFTR), and AQP9 co-localize in the apical membrane of principal cells of the epididymis and the vas deferens, and that both NHERF1 and CFTR co-immunoprecipitate with AQP9. Overlay assays revealed that AQP9 binds to both the PDZ1 and PDZ2 domains of NHERF1, with an apparently higher affinity for PDZ1 versus PDZ2. Pull-down assays showed that the AQP9 COOH-terminal SVIM motif is essential for interaction with NHERF1. Functional assays on isolated tubules perfused in vitro showed a high permeability of the apical membrane to glycerol, which is inhibited by the AQP9 inhibitor, phloretin, and is markedly activated by cAMP. The CFTR inhibitors DPC, GlyH-101 and CFTRinh-172 all significantly reduced the cAMP-activated glycerol-induced cell swelling. We propose that CFTR is an important regulator of AQP9 and that the interaction between AQP9, NHERF1, and CFTR may facilitate the activation of AQP9 by cAMP.

Epithelial cells lining the lumen of the excurrent duct of the male reproductive tract create a luminal environment that is optimal for sperm maturation and storage. The composition of the luminal fluid is progressively modified and is tightly regulated during transit from the testicular seminiferous tubules, into the efferent ducts, epididymis, and vas deferens (1–10). Significant water reabsorption leading to a marked increase in sperm concentration and luminal hypertonicity occurs in the epididymis (5, 11–13). In addition, glycerol, a metabolic substrate for epididymal sperm, is accumulated in the lumen of the distal epididymis (14). In the more distal regions of the epididymis and in the vas deferens, water secretion driven by cystic fibrosis transmembrane conductance regulator (CFTR)2-dependent chloride transport occurs and controls the fluidity of the luminal content (15, 16). Although the epididymis is among the most seriously affected organs in cystic fibrosis (CF), very little is known about the mechanisms that lead to the marked decrease in male fertility that occurs in this disease. Cystic fibrosis is one of the leading causes of male infertility (15, 17, 18). CFTR plays a critical role in the anatomy and function of the epididymis and vas deferens. A large number of men with CF have no vas deferens, and/or absence or atrophy of some regions of the epididymis (19, 20). It was originally proposed that these abnormalities were the consequence of defective embryonic development. However, recent studies have indicated that dysfunction of the epididymis and vas deferens in patients with cystic fibrosis might be the results of a progressive atrophy of these tissues that may occur after birth and reach maximum intensity at adult age (21, 22). These studies suggest that prevention strategies could be developed to help the CF-affected male population preserve their reproductive function.

In a variety of epithelia, water channels (aquaporins) are involved in transepithelial bulk water flow driven by an osmotic gradient (reviewed in Ref. 23). In mammals, aquaporins are

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†To whom correspondence should be addressed: Program in Membrane Biology, Simches Research Center, Massachusetts General Hospital, 185 Cambridge St., Suite 8204, Boston, MA 02114. Tel.: 617-726-5785; Fax: 617-643-3182; E-mail: sbreon@partners.org.

‡The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; NHERF1, Na+/H+ exchanger regulatory factor 1; cpt-CAMP, chlorophenylthio cAMP; IP, immunoprecipitation; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BBM, brush border membrane; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; aa, amino acid(s); DPC, diphenylecarbamyl chloride.

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divided into two subgroups based on their permeability characteristics: the strict “aquaporins” (AQP0, 1, 2, 4, 5, 6, and 8) are selective for water and the “aquaglyceroporins” (AQP3, 7, 9, and 10) are permeable to neutral solutes in addition to water. AQP11 and AQP12 have recently been identified and are more distantly related to the other members of the aquaporin family (24). Aquaporins and aquaglyceroporins show a wide range of distribution in organs that are actively involved in water movement (25–29). AQP9 has been identified as the major aquaglyceroporin in the everted duct of the male reproductive tract, the liver, and peripheral leukocytes (30–36). In the male reproductive system, it is constitutively expressed in the apical stereocilia of principal cells along the entire length of the epididymis and vas deferens, as well as in the apical membrane of nonciliated cells of the efferent ducts (32). This aquaglyceroporin allows passage of a wide range of solutes, including glycerol, urea, mannitol, and sorbitol, in addition to water (35). Thus, AQP9 provides a potential route for transepithelial fluid and solute transport in the epididymis. The promoter region of AQP9 contains a putative steroid hormone receptor-binding site (35), and sex-linked differences in AQP9 expression were reported in the liver (37). Androgens control AQP9 expression in the adult epididymis (30, 33, 38), and Aqp9 mRNA increases markedly during the first 4 weeks of postnatal development (31). However, the acute regulation of AQP9 function has not been well characterized. The presence of a putative PDZ (PSD-95, Drosophila discs large protein, ZO-1) binding motif, SVIM, in the COOH terminus of AQP9 indicates the potential intervention of PDZ proteins in its regulation. PDZ proteins are scaffolding proteins that facilitate the association of multiprotein complexes, a process that is essential for the phosphorylation of some transporters, channels, and receptors (39, 40). NHERF1 (Na/H exchanger regulatory factor; SLC9A3R1) is a major apical PDZ protein that contains three protein interaction domains: PDZ domain 1 (PDZ-1), PDZ domain 2 (PDZ-2), and a sequence located in the COOH terminus that binds to the family of Merlin/Ezrin/Radixin/Moesin (ERM) proteins (41–43). NHERF1 is involved in the cAMP regulation of a variety of transporters, including Na+/H+ exchanger type 3 (NHE3), CFTR, Na+/P+, cotransporter IIa (Npt2 or NaP1, Iia) (reviewed in Refs. 39, 40, 44, and 45)), and ROMK (46).

Water transport and solute transport represent crucial events in the establishment and maintenance of male fertility, and we postulated that CFTR might be involved in their regulation. The present study is aimed at characterizing the functional contribution of AQP9 to apical glycerol permeability and at determining whether the apical PDZ protein NHERF1, and the PDZ-binding protein, CFTR, could participate in its regulation.

**EXPERIMENTAL PROCEDURES**

Functional Studies on Epididymal Tubules Perfused in Vitro—Epididymal tubules were dissected from the initial segments of the epididymis in a cold preservation solution containing 56 mM Na₂HPO₄, 13 mM NaH₂PO₄, and 140 mM sucrose, as described previously (47). They were then transferred into a perfusion chamber mounted on the stage of an Olympus IMT-2 inverted microscope, and peritubular and luminal perfusions were performed (solutions in Table 1). The basolateral solution composition was based on normal plasma values, and the apical solution was based on previous epididymal micropuncture studies (5). After an initial control period, the apical membrane permeability to glycerol was estimated from the initial rate of increase in cellular volume induced upon isotonic replacement of either 60 or 120 mM raffinose (an impermeant solute in the epididymal tubule) with glycerol. Digital images of perfused tubules were captured at 15- or 30-s intervals, as described in the text, using a Nikon Coolpix 995 camera and were analyzed using IPLab software (Scananalytics, Fairfax, VA). For each time point, the height of epithelial cells was measured at 5–6 different locations along the tubule, and the values were averaged. Cell volume was assessed from these values and expressed as percentage of initial control volume, as we have previously published for kidney proximal tubules (48). Initial rates of cell swelling were determined from four cell volume values measured at 15-s intervals during the first minute of glycerol exposure. The effects of 500 μM phloretin, an AQP9 inhibitor, or 100 μM chlorophenylthio cAMP (cpt-cAMP) on glycerol-induced cell swelling were examined. We also examined the effects of three different CFTR inhibitors, DPC (500 μM), GlyH–101 (25 μM), and CFTRinh–172 (5 μM). GlyH–101 and CFTRinh–172 are previously characterized specific CFTR inhibitors kindly provided by Alan Verkman (University of California, San Francisco) (49, 50). Statistical analysis was performed using the Student’s t test for paired or unpaired experiments, as indicated in the text.

**Antibodies and Peptides**—An affinity purified rabbit polyclonal antibody was raised against a peptide corresponding to the last 15 amino acids (PSENLEKHELSVIM) of the COOH-terminal tail of rat AQP9 (35). This antibody has been fully characterized previously (32, 33) and was used in this study for immunocytochemistry and for some immunoprecipitation (IP) assays. An affinity purified anti-rat AQP9 antibody raised in chicken (Chemicon International; Temecula, CA) was also used for Western blotting of material immunoprecipitated using our rabbit anti-AQP9 antibody. An affinity purified chicken polyclonal antibody was raised against a GST-NHERF1 fusion protein corresponding to amino acids 270–358 (IC270), which we used previously for the generation of a polyclonal

### TABLE 1

| Composition of luminal and basolateral solutions | Control luminal solution | Glycerol solution | Basolateral solution |
|-----------------------------------------------|-------------------------|------------------|---------------------|
| NaCl                                          | 55                      | 55               | 100                 |
| KCl                                           | 5                       | 5                | 5                   |
| MgSO₄                                         | 1.2                     |                  |                     |
| Na₂HPO₄                                       | 1                       | 1                | 1                   |
| Glucose Cl                                    | 10                      |                  |                     |
| Raffinose                                     | 120 (0 or 60)           |                  |                     |
| CaCl₂                                         | 1.8                     | 1.8              | 1.8                 |
| MgCl₂, H₂O                                    | 1.2                     | 1.2              |                     |
| Sodium acetate                                | 4                       | 4                | 4                   |
| Na₂ citrate                                   | 1                       | 1                | 1                   |
| Glucose                                       | 5.5                     | 5.5              | 5.5                 |
| Alamine                                       | 6                       | 6                | 6                   |
| Na₂HPO₄                                       | 3                       | 3                | 3                   |
| NaHCO₃                                       | 5                       | 5                | 25                  |
| Glycerol                                      | 120 (0 or 60)           |                  |                     |
| Sodium cyclamate                              | 25                      |                  | 25                  |
| Osmolarity (mOsm/kg H₂O)                      | 331                     | 334              | 297                 |
| pH                                           | 6.85                    | 6.83             | 7.41                |
rabbit antibody (41). A peptide corresponding to the last 15 amino acids of AQP9 was generated in the Massachusetts General Hospital Peptide/Protein Core Facility, and some of the peptide was biotinylated. Three different anti-CFTR antibodies were used for immunofluorescence and Western blot detection. AME-4991 is the whole serum of a previously characterized antibody raised in rabbit against a synthetic 13-residue peptide of the carboxyl terminus of rat CFTR (51). A commercial rabbit affinity purified antibody against amino acid residues 1468–1480 of human CFTR (ACL-006 from Alomone) and a monoclonal antibody against amino acids 1377–1480 of human CFTR (Clone 24-1, MAB25031 from R&D Systems) were also used.

**Immunofluorescence Microscopy**—Sexually mature male Sprague-Dawley rats were anesthetized with Nembutal (7.5 mg/100 g body weight intraperitoneal; Abbott Laboratories, North Chicago, IL) and perfused via the left ventricle with PBS (0.9% NaCl in 10 mM sodium phosphate buffer, pH 7.4) followed by a fixative containing 4% paraformaldehyde, 10 mM sodium phosphate, 75 mM lysine, and 5% sucrose in 0.1 M sodium phosphate buffer, as described previously (32, 33), or with PBS containing 2% paraformaldehyde (for CFTR labeling). Epithelium and vas deferens were cryoprotected in 30% sucrose in PBS, containing 2% paraformaldehyde (for CFTR labeling). Epipidymis and vas deferens were cryoprotected in 30% sucrose in PBS, mounted for cryosectioning in Tissue-Tek OCT compound 4583 (Sakura Fintek USA, Inc., Torrance, CA), and quick frozen. Sections were cut at a thickness of 5 µm using a Reichert Jung 2800 Frigocut cryostat (Leica Microsystems, Inc., Bannockburn, IL) and picked up onto Superfrost/Plus microscope slides (Fisher Scientific). For indirect immunofluorescence microscopy, sections were hydrated for 15 min in PBS and treated for 4 min with 1% SDS in PBS, an antigen retrieval technique that we have previously described (52). Sections were washed in PBS 3 times for 5 min and then blocked in 1% bovine serum albumin/PBS for 15 min. Affinity purified rabbit anti-AQP9 antibody was applied at a dilution of 1:3200 in a moist chamber for 90 min at room temperature or overnight at 4 °C. Sections were washed in high salt PBS (PBS containing 2.7% NaCl) twice for 5 min and once in normal PBS. Goat anti-rabbit IgG coupled to CY3 was then applied for 1 h at room temperature followed by washes as above. Sections were double-stained by subsequent incubation with anti-NHERF1 antibody diluted 1:50 followed by donkey anti-chicken IgG conjugated to fluorescein isothiocyanate, or with anti-CFTR antibody MAB25031 diluted 1:10, followed by goat anti-mouse IgG conjugated to fluorescein isothiocyanate. Double labeling was also performed using anti-AQP9 chicken antibody diluted 1:50 followed by anti-CFTR AME-4991 antibody diluted 1:50.

Slides were mounted in Vectashield medium (Vector Laboratories, Inc., Burlingame, CA). Digital images were acquired using a Nikon Eclipse 800 epifluorescence microscope (Nikon instruments, Inc., Melville, NY) using an Orca 100 CCD camera (Hamamatsu, Bridgewater, NJ), analyzed using IPLab scientific imaging processing software (Scanalytics, Inc., Fairfax, VA), and imported into Adobe Photoshop image editing software (Adobe Systems Inc., San Jose, CA).

**Apical Membrane Preparation**—Epithelial cell apical membranes were isolated using the brush border membrane (BBM) Mg²⁺ precipitation technique, as previously described (32, 33). We have shown previously that AQP9 is significantly enriched in epididymal BBM (32). Protein concentration was determined using the bicinchoninic acid assay (Pierce Biotechnology) using albumin as standard.

**IP and Co-IP Assays**—Anti-AQP9 rabbit antibody was conjugated to magnetic beads (Dynabeads Protein A, Invitrogen) according to the manufacturer’s protocol. The epididymal BBM preparation (250 µg) was pre-cleared by two consecutive 30-min incubations with non-conjugated magnetic beads. Immunoprecipitation assays were performed in 1 ml of IP buffer (1% Triton X-100, 100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.5 mM IGEPAL CA-630, 10% glycerol, 1% bovine serum albumin, complete protease inhibitors) for 2 h at 4 °C. After three washes in 1 ml of IP buffer, beads were resuspended in 50 µl of Laemmli reducing sample buffer, and incubated at room temperature for 45 min. Beads were captured using a magnetic particle concentrator (Invitrogen), and eluates were subjected to SDS-PAGE, as described below. For some experiments, the anti-AQP9 antibody was preincubated with the non-biotinylated AQP9 peptide prior to immobilization on the beads. In separate experiments, CFTR and AQP9 co-IP assays were performed using rabbit anti-CFTR antibody (Alomone) and our rabbit anti-AQP9 antibody, which were bound and cross-linked to immobilized protein A using the Seize X Protein A immunoprecipitation kit (Pierce). This procedure allowed for Western blot detection of proteins in the IP material using antibodies raised in the same species as that used for the IP. Total proteins from rat epididymis and lung were isolated using the ProFound lysis buffer (Pierce) complemented with protease inhibitors. CFTR co-IP was performed by incubating the immobilized anti-CFTR antibody sequentially with 1 mg of lung extract for 4 h, then with 1 mg of epididymis extract enriched with BBM overnight. This sequence was reversed to perform the AQP9 co-IP. After three washes, proteins were eluted in NuPAGE LDS sample buffer (Invitrogen) with reducing agent and protease inhibitors, incubated for 45 min at 23 °C, and analyzed by Western blotting.

**Immunoblotting (SDS-PAGE and Western Blotting)**—Total epididymis homogenates, BBM samples, or IP eluates were diluted in sample buffer, and loaded onto Tris glycine polyacrylamide 4–20% gradient gels (Lonza, Rockland, ME). 4–12% NuPAGE gels (Invitrogen) were used for the analysis of CFTR/AQP9 co-IPs. After SDS-PAGE separation, proteins were transferred onto Immuno-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dry milk and then incubated overnight at 4 °C with the primary antibody (anti-AQP9, anti-NHERF1, or -CFTR) diluted in TBS containing 2.5% milk. After three washes in TBS containing 0.1% Tween 20 (TBST), and a 15-min block in 5% milk/TBS, membranes were incubated with secondary antibodies (either goat anti-rabbit IgG or goat anti-chicken IgG) conjugated to horseradish peroxidase for 1 h at room temperature. After five further washes, antibody binding was detected with the Western Lightning Chemiluminescence reagent (PerkinElmer Life Sciences) and Kodak imaging films.

**Phosphatase Assay**—330 µg (50 µl) of epididymis total homogenate was incubated with 100 µl of 1 mM Tris, 50 mM...
Tris-HCl, pH 7.5, for 10 min at 30 °C. 30 Units (30 \mu l) of calf intestine alkaline phosphatase (Calbiochem, Darmstadt, Germany) was then added (water was added in the control sample) and the solution was incubated for 15 min at 30 °C. Dephosphorylation was terminated by the addition of 180 \mu l of Laemmli sample buffer (2 times). 30 \mu l of each sample (containing 27 \mu g of protein) were then subjected to electrophoresis and Western blot, as described above.

Preparation of Recombinant NHERF1—Human full-length NHERF1 (amino acids (aa) 1–358) and NHERF1 truncated fusion proteins containing PDZ1 (aa 11–97), PDZ2 (aa 149–236), PDZ1 and PDZ2 (aa 11–236), PDZ2 and the COOH-terminal portion (aa 149–358), and NHERF1 lacking both PDZ domains (aa 270–358) were amplified by PCR, subcloned into the BamHI-NotI sites of pGEX4T (Amersham Biosciences), and expressed in Escherichia coli, as described previously (43). After a first step of purification using glutathione-Sepharose 4B (Amersham Biosciences), GST fusion proteins were loaded onto Tris glycine polyacrylamide 4–20% gradient gels (PAGEr Duramide Precast Gels, 4–20% Tris glycine gels, Cambex, Rockland, ME). After SDS-PAGE separation, GST proteins were transferred onto a PVDF membrane. After staining with Ponceau stain (Ponceau S Stain, Boston Bioproducts, Ashland, MA) bands of interest were eluted from PVDF membrane in Triton/SDS elution buffer 50 mM Tris-Cl, pH 9, 2% (w/v), 1% (v/v) Triton X-100, 0.2 ml/cm² of membrane). These pure protein preparations were then stored at −20 °C until use.

Overlay Assays—Purified NHERF1 GST fusion proteins were submitted to electrophoresis and transferred onto a PVDF membrane, as described above. Membranes were blocked in 10% nonfat dry milk in TBST, followed by two sequential 1-h incubations, the first with avidin (Avidin from egg white, Sigma) at 0.2 mg/ml and the second with biotin (D-Biotin, Sigma) at 0.1 mg/ml to block avidin and biotin sites. Membranes were incubated with biotinylated COOH terminus AQP9 peptide at a concentration of 25 \mu g/ml overnight at 4 °C, followed by washes. Membranes were then incubated with avidin conjugated to horseradish peroxidase at a dilution of 1:2000 (ExtrAvidin Peroxidase conjugate, Sigma). Probe binding was detected using enhanced chemiluminescence (PerkinElmer Life Sciences) and Kodak X-Omat blue XB-1 films. TBS was used for all washes and incubations. After development, the membrane was washed in TBS and stained with Coomassie Blue to confirm the purity of the constructs.

Preparation of AQP9 COOH-terminal Constructs—A cDNA fragment (base pairs 1055–1163) of the rat AQP9 cDNA (GenBank™ accession number AF016406, provided by Dr. Matthias Hediger), corresponding to the cytosolic COOH-terminal portion (aa MKAPSENNLHELSVIM) of rat AQP9 (35) was amplified by PCR. The fragment was then digested and inserted into PstI and SpeI sites of pET41a expression vector (Novagen). AQP9-COOH-ter was GST epitope-tagged at the NH₂ terminus. In some constructs, the SVIM motif of the GST-AQP9-COOH-ter was mutated to GGGG and SAKH. The sequence, SAKH, corresponds to the last four amino acids of the B2 subunit of the V-ATPase, which has been shown not to interact with NHERF1 (53), and was, therefore, designed as a negative control. SVIM was also deleted (GST-AQP9-truncated) to prevent the formation of non-specific complexes.
obtain a truncated protein. Mutations were performed by using a QuikChange Site-directed mutagenesis kit (Stratagene). For protein expression, BL21(DE3) pLysS competent cells were transformed with the different constructs. Fidelity of the constructs was confirmed by DNA sequence analysis.

Small-scale overnight bacteria cultures were started from glycerol stocks and were grown in LB broth at 37 °C. Two ml of the overnight culture was added to 200 ml of LB broth (1:100 dilution) containing 30 μg/ml kanamycin. When cultures reached an A$_{600}$ of 0.4–0.6, they were induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C. The bacteria were washed and resuspended in 25 ml of cold PBS containing 2.5 mM EDTA. Lysis was performed using a French press, at 1000 pounds/inch$^2$. The lysates were then centrifuged at 10,000 × g for 30 min at 4 °C. Supernatants were aliquoted and stored at −80 °C.

**Pull-down Assays**—GST-AQP9 fusion proteins were immobilized onto glutathione-Sepharose beads (Amersham Biosciences). For pull-down of NHERF1, 150 μg of rat epididymis brush border membranes were resuspended in a Tris buffer (20 mM Tris, 0.1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1% IGEPAL, pH 7.40) and incubated with the immobilized GST proteins in a rotator for 2 h at 4 °C. Beads were recovered by centrifugation, washed extensively, and resuspended in Laemmli buffer. Samples were boiled for 5 min and then subjected to SDS-PAGE and immunoblot analysis for NHERF1, using the affinity purified chicken anti-NHERF1 antibody IC270. Pull-down assays were also performed using kidney lysate as a source of abundant NHERF1 that does not contain endogenous AQP9.

**RESULTS**

**AQP9-dependent Apical Membrane Glycerol Permeability**—Isolated epididymal tubules were perfused with a control solution containing 120 mM raffinose, a solute to which epididymis epithelial cells are impermeant. The epithelial cell layer could be visualized and distinguished from the surrounding connective tissue and muscle cells (Fig. 1A). After a 5-min control period, raffinose was replaced by 120 mM glycerol for 5 min (Fig. 1B), followed by a post-control period. Glycerol permeability was estimated from the increase in cell volume induced by isotonic replacement of raffinose by glycerol. Cell volume was estimated from the height of epithelial cells measured at 5 different locations along the tubule (Figs. 1, C and D), as we have described previously (48). Significant cell swelling was observed upon glycerol addition indicating high permeability of the epididymal apical membrane to this neutral solute (Fig. 2A, top trace). On average, the initial rate of cell swelling was

![Figure 3](image3.png)

**Figure 3.** Effect of cAMP on glycerol-induced cell swelling. Left bars, control tubules subjected to two consecutive pulses of 60 mM glycerol (GLY60). Identical initial rates of cell swelling were detected. Middle bars, tubules were subjected to a first glycerol pulse (GLY60) followed by addition of cpt-cAMP for 5 min and a second pulse of glycerol still in the presence of cpt-cAMP (GLY60 cAMP). CAMP induced a significant increase in the initial rate of cell swelling. Right bars, tubules were subjected to a first glycerol pulse (GLY60) followed by addition of cpt-cAMP for 3 min and a second pulse of glycerol in the presence of cpt-cAMP and phloretin (GLY60 cAMP phloretin). Phloretin inhibited CAMP-activated glycerol-induced cell swelling. Data are mean ± S.E., *, p < 0.02; **, p < 0.0005, Student’s t test for paired experiments.

![Figure 4](image4.png)

**Figure 4.** Cryosections of rat epididymis and vas deferens double-stained for AQP9 and NHERF1. AQP9 (red) and NHERF1 (green) are located in the apical membrane of principal cells from the initial segments (A–C), caput (D–F), cauda (G–I) regions of the epididymis, and in the vas deferens (J–L). Merge panels (C, F, I, and L) show co-localization of AQP9 with NHERF1 (yellow). In the cauda epididymidis, clear cells, negative for AQP9 also express abundant NHERF1 in their apical membrane (green staining in I). Bars = 50 μm.
50.6 ± 10%/min (n = 8). Addition of the AQP9 inhibitor phloretin (500 μM) into the luminal fluid significantly reduced the initial rate of cell swelling to 7.5 ± 3.7%/min (Fig. 2, A, bottom trace and B, n = 10). These results indicate significant AQP9-dependent apical membrane permeability to glycerol.

Effect of cAMP on AQP9-dependent Glycerol Permeability—To test for the effect of cAMP on AQP9-dependent glycerol permeation, lower glycerol concentrations of 60 mM were used to facilitate the detection of any variations in the initial rate of cell volume changes, by slowing down the overall speed of the process. First, we showed that two consecutive pulses of glycerol, separated by a washout period of 10 min, induced identical cell swelling (Fig. 3). The same protocol was then applied but 5 min prior to performing the second glycerol pulse, a permeant analogue of cAMP (cpt-cAMP, 100 μM) was added into the luminal perfusate. Whereas cpt-cAMP alone did not induce any change in basal cell volume (data not shown), the initial rate of glycerol-induced cell swelling was markedly increased by cAMP (83.3 ± 25.8%/min), compared with control (17.8 ± 5.3%/min; p < 0.02, n = 5) (Fig. 3). Importantly, phloretin completely inhibited the cAMP-activated, glycerol-induced cell swelling (1.53 ± 1.5 versus 30.65 ± 4.33%/min, p < 0.0005, n = 6) (Fig. 3).

Co-localization of AQP9 with NHERF1 in the Apical Membrane of Principal Cells—Double immunofluorescence labeling revealed abundant expression of the PDZ protein NHERF1 in the apical membrane of principal cells of all regions of the epididymis and vas deferens, where it co-localized with AQP9 (Fig. 4). In the distal epididymis (cauda), clear cells were negative for AQP9 as we have previously shown (32), but they showed significant apical membrane and weak intracellular NHERF1 staining (Fig. 4, G–I).

Co-immunoprecipitation of NHERF1 with AQP9—As shown in Fig. 5A, NHERF1 was abundant in the epididymis BBM preparation, and it was co-immunoprecipitated from rat epididymal BBM using our anti-AQP9 antibody (IP AQP9) but not with protein A-conjugated beads (IP Control). Interestingly, two bands at around 50 kDa were detected by the antibody. Incubation of total epididymis homogenates with intestine alkaline phosphatase reduced the intensity of the upper band in the doublet and increased the lower band intensity (Fig. 5B). These results indicate that the upper band represents phosphorylated NHERF1 and that the phosphorylated form of NHERF1 is predominant in the epididymis preparation under our experimental conditions. Thus, the two bands detected in the AQP9 co-immunoprecipitated material shown in Fig. 5A represent the phosphorylated and non-phosphorylated forms of NHERF1.

The COOH-terminal Tail of AQP9 Interacts with NHERF1—To determine the contribution of the AQP9 COOH terminus to the interaction with NHERF1, co-immunoprecipitation assays were repeated after preincubation of the AQP9 antibody-conjugated beads with an AQP9 peptide containing the SVIM motif. Western blot for NHERF1 was first performed using the affinity purified chicken antibody (Fig. 6, top panel) and the same membrane was subsequently blotted for AQP9 (Fig. 6, bottom panel). A progressive displacement of AQP9 was observed when the AQP9 antibody was preincubated with increasing concentrations of the AQP9 peptide (bottom panel: compare lane 2 (no peptide) with lanes 3 and 4). In lane 4, a complete displacement of AQP9 by the peptide was achieved. As shown in the top panel, the amount of NHERF1 in the AQP9-IP material was directly proportional to the level of displacement of AQP9 by the AQP9 peptide, indicating that the AQP9 peptide containing the SVIM motif is more efficient in immunoprecipitating NHERF1 than the holo-AQP9 protein. This might be due to a better accessibility of the SVIM motif located on the peptide for interaction with NHERF1, versus the holo-AQP9 protein when bound to the antibody. No AQP9 or NHERF1 were detected in the control lane using protein A beads alone (Fig. 6, lane 1). This result supports our hypothesis that the last amino acids of AQP9, which include the “SVIM” motif, mediate the interaction of AQP9 with NHERF1.

Direct Interaction between the COOH Terminus Tail of AQP9 and NHERF1 PDZ Domains—Overlay assays using purified NHERF1 GST fusion proteins subjected to electrophoresis and transferred onto PVDF membrane revealed a specific and direct interaction between the AQP9 peptide and both PDZ domains of NHERF1 (Fig. 7). Whereas no AQP9 binding was
detected in the control lane containing GST alone, or with the NHERF1 construct lacking both PDZ domains (GST-IC270), significant amounts of AQP9 were detected in lanes containing PDZ1 domain (GST-PDZ1), both PDZ domains together (GST-PDZ1-PDZ2), and the entire length of NHERF1 (GST-NHERF1). A lower amount of AQP9 was detected in the lane containing the PDZ2 domain (GST-PDZ2) indicating lower affinity of AQP9 for PDZ2 compared with PDZ1. No binding was detected with the GST-IC149 fusion protein, indicating negative cooperativity between the PDZ2 domain and the remaining COOH terminus portion of NHERF1. The higher affinity of AQP9 for PDZ1 compared with PDZ2 was confirmed by using sequential dilutions of the NHERF1 PDZ1 and PDZ2 constructs (data not shown).

The SVIM Motif of AQP9 Is Essential for the AQP9-NHERF1 Interaction—Pull-down assays were performed from epididymal BBM (Fig. 8, left panel) or kidney cortex lysate (Fig. 8, right panel) using GST-AQP9 fusion proteins corresponding to either the last 20 amino acids of the COOH terminus tail of AQP9 (GST-AQP9-COOH-term) or a truncated COOH terminus tail in which the SVIM motif has been deleted (GST-AQP9-truncated). Kidney lysate was used as a source of abundant NHERF1 that does not contain endogenous AQP9, which might have competed away the interaction of NHERF1 with immobilized AQP9 fusion protein in

![FIGURE 6. The COOH terminus of AQP9 mediates co-immunoprecipitation of NHERF1. The anti-AQP9 antibody was preincubated with the AQP9 peptide containing the SVIM motif prior to co-immunoprecipitation using epididymal BBM. Top panel, immunoblot for NHERF1. Bottom panel, the same membrane was re-stained for AQP9. A progressive displacement of AQP9 was observed when the anti-AQP9 antibody was preincubated with increasing concentrations of the AQP9 peptide (compare lane 2 (no peptide) with lanes 3 and 4). In lane 4, a complete displacement of AQP9 by the peptide was achieved. The amount of co-immunoprecipitated NHERF1 is directly proportional to the level of displacement of AQP9 by the AQP9 peptide. IP Control, protein A-beads alone; IP AQP9 IgG, no peptide; IP AQP9 + peptide, IP after preincubation with the AQP9 peptide; BBM, protein extract from epididymal brush border membranes (lane 6) and no sample (lane 5).]

![FIGURE 7. Direct interaction between the COOH terminus of AQP9 and NHERF1. Top panel, overlay of biotinylated peptide containing the last COOH-terminal 15 amino acids of AQP9 (30 μg/ml) onto membranes containing various constructs of GST-NHERF1. Binding of AQP9 peptide was detected in lanes containing PDZ1 (GST-PDZ1) or PDZ2 (GST-PDZ2) domains of NHERF1, a NHERF1 construct containing both PDZ domains (GST-PDZ1-PDZ2), and the full-length of NHERF1 (GST-NHERF1). No binding was detected in the control lane containing GST alone (GST), or lanes containing a NHERF1 construct lacking both PDZ domains (GST-IC270) or lacking PDZ1 domain (GST-IC149). A higher amount of AQP9 was detected in the lane containing PDZ1 compared with PDZ2, indicating higher affinity for PDZ1. Bottom panel, the same membrane was stained with Coomassie Blue to illustrate the relative quantity and size of the immobilized NHERF1 fusion proteins.]

![FIGURE 8. Pull-down of NHERF1 with GST-AQP9 constructs. Epididymal BBM (left panel) or kidney lysate (right panel) were incubated with Sepharose beads conjugated with GST-AQP9 fusion proteins. NHERF1 was pulled down from both preparations using the AQP9 construct corresponding to the last 20 amino acids of the COOH-terminal tail of AQP9 (GST-AQP9-c-term). In contrast, no NHERF1 was detected in the samples incubated with the GST-AQP9 COOH terminus construct lacking the SVIM motif (GST-AQP9-truncated). A lower molecular band, corresponding to GST-AQP9 was also detected with the anti-NHERF1 antibody, which was raised using a GST-NHERF1 construct, and therefore detects GST, in addition to NHERF1. This band provides visualization of the amount of GST-AQP9 that was used for the pull-down assay, and serves as a loading control. Abundant NHERF1 was detected in total kidney lysate.]
the epididymis BBM preparation. NHERF1 was pulled down from both epididymis and kidney preparations using the full-length AQP9 COOH terminus fusion protein preparations. In contrast, no NHERF1 was detected in the sample pulled down using AQP9 constructs in which the SVIM motif had been completely deleted (Fig. 8) or had been replaced by either GGGG or the non-related motif SAKH (corresponding to the last amino acid of the B2 subunit of the V-ATPase, which does not interact with NHERF1) (53) (data not shown). These results demonstrate that the SVIM motif is essential for the interaction between the COOH terminus tail of AQP9 and native NHERF1.

**FIGURE 9. Cryosections of rat epididymis double-stained for AQP9 and CFTR.** AQP9 (red) and CFTR (green) are located in the apical membrane of principal cells from the initial segments (A–C), and cauda (D–F) regions of the epididymis. Merge panels (C and F) show partial co-localization of AQP9 with CFTR at the base of the stereocilia of principal cells (yellow). Insets in panels D–F are larger magnification of the apical pole of the cells located in the boxes. Bars = 25 μm.

**FIGURE 10. Western blot detection of CFTR in rat epididymis and lung.** A, total homogenates from rat lung (40 μg) and epididymis (40 μg), and epididymal BBM (20 μg) were loaded onto each lane. Western blot using anti-CFTR antibody ACL-006 from Alomone revealed a single band at around 140 kDa. A significantly stronger band was detected in the BBM compared with total epididymis homogenate, showing enrichment of CFTR in the apical membrane preparation. B, complete inhibition of the staining was detected after preincubation of the antibody with its antigenic peptide.

**DISCUSSION**

We and others have shown that AQP9 is the major apical aquaporin in the excurrent duct of the male reproductive system (30–34). AQP9 is highly expressed in the apical membrane of principal cells of the epididymis (32). Throughout the epididymis, considerable water reabsorption occurs, leading to a significant increase in spermatozoa concentration (5, 12, 54–57).
In the distal regions, water secretion driven by CFTR-dependent chloride transport regulates the final fluidity of the luminal environment in which sperm mature and are stored (15). A previous study showed AQP9-dependent transepithelial water transport in the distal epididymis perfused in vivo (58). Interestingly, AQP9 is not only permeant to water, but it also allows permeation of neutral solutes such as glycerol (35). Glycerol is accumulated in the lumen of the epididymis (14), and may serve as a metabolic substrate for epididymal sperm. Thus, both the neutral solute permeability and water permeability of AQP9 might represent important functions for the preservation and storage of sperm in the lumen of the epididymis.

We have previously shown that, in contrast to some other apical aquaporins, AQP9 does not recycle between the plasma membrane and intracellular vesicles (32). For example, in the kidney AQP2 is accumulated in the apical membrane by modulation of vesicle trafficking after cAMP stimulation (59), and in the airway epithelium cAMP induces the internalization of AQP5 (60). Thus, the regulation of AQP9 by cAMP that we show here in the proximal epididymis, and that others have described in the distal epididymis (58) seems to occur without the intervention of recycling mechanisms. The direct interaction between AQP9 and NHERF1 that we reveal in the present study strongly indicates that NHERF1 might serve to anchor and stabilize AQP9 in the plasma membrane, as was shown for other resident membrane proteins (39, 40, 44). NHERF1 is a major PDZ-containing adapter protein that facilitates multi-protein complex formation, an essential step for the phosphorylation and regulation of a growing number of transporters, channels, and receptors (39, 40, 43, 44, 46, 61). NHERF1 was first identified as an essential cofactor for the cAMP regulation of NHE3 in the kidney (62). NHERF1 participates in the formation of a complex that contains NHE3 and the protein kinase A-anchoring protein, ezrin, which facilitates protein kinase A phosphorylation of NHE3 (63). Whereas we propose that NHERF1 may participate in the cAMP activation of AQP9 in the epididymis by analogy with its role in regulating other proteins, a direct activation of AQP9 by cAMP remains possible. AQP9 contains several putative serine and tyrosine phosphorylation sites in its cytoplasmic COOH- and NH2-terminal tails, and phosphorylation events mediated by protein kinase A, protein kinase G, or protein kinase C might be responsible for the regulation of AQP9 permeability. Dissection of the phosphorylation sites on AQP9, their physiological role, and the potential participation of NHERF1 in this process will require further studies.

In the present study, we show that a significant portion of NHERF1 is phosphorylated under basal conditions in the epididymis, and that both the phosphorylated and non-phosphorylated forms of NHERF1 are co-immunoprecipitated with
AQP9. Interestingly, phosphorylation of NHERF1 was shown to be a key factor in the regulation of CFTR, which binds to both PDZ domains of NHERF1 (64, 65). The formation of a complex that contains two CFTR molecules for one NHERF1 increases the open probability of CFTR (reviewed in Ref. 45). Phosphorylation of the PDZ2 domain of NHERF1 by protein kinase C disrupts its interaction with CFTR, keeping the interaction between CFTR and PDZ1 intact, but preventing the stimulatory effect of NHERF1 (66). It was proposed that prevention of the bivalent coupling of CFTR following phosphorylation of NHERF1 would switch CFTR from being an open chloride channel to a regulatory protein (46). A similar mechanism was thought to regulate the functional coupling between CFTR and ROMK, via another member of the NHERF family, NHERF2 (46).

Interestingly, Cheung et al. (58) showed that CFTR conferred cAMP-dependent activation of AQP9 in Xenopus oocytes and that inhibition of CFTR by lonidamine reversed the cAMP activation of AQP9 in the intact cauda epididymis. In the present study, we showed that, similarly to CFTR, AQP9 binds to both PDZ domains of NHERF1. CFTR is also expressed in the apical step that helps control the final fluidity of the luminal content.

AQP9-dependent cAMP-activated glycerol permeability. In conclusion, the present study shows that AQP9 specifically interacts with both PDZ domains of NHERF1 via its SVIM COOH-terminal motif. We also show that CFTR is present in the AQP9-immunoprecipitated protein complex and that it participates in the regulation of AQP9 function. In addition to AQP9, other members of the aquaporin family, including AQ2 and AQP4, also contain putative COOH-terminal PDZ binding motifs. The interaction between aquaporins and PDZ proteins could, therefore, represent a widespread mechanism by which water and neutral solute transport are regulated.

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