# Aquaporin 3 cloned from *Xenopus laevis* is regulated by the cystic fibrosis transmembrane conductance regulator[1]

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**Abstract The cystic fibrosis transmembrane conductance regulator (CFTR) is essential for epithelial electrolyte transport and has been shown to be a regulator of epithelial $Na^+$, $K^+$, and $Cl^-$ channels. CFTR also enhances osmotic water permeability when activated by cAMP. This was detected initially in *Xenopus* oocytes and is also present in human airway epithelial cells, however, the mechanisms remain obscure. Here, we show that CFTR activates aquaporin 3 expressed endogenously and exogenously in oocytes of *Xenopus laevis*. The interaction requires stimulation of wild type CFTR by cAMP and an intact first nucleotide binding domain as demonstrated for other CFTR–protein interactions.** 

*Key words:* Aquaporin 3; Cystic fibrosis transmembrane conductance regulator; Cystic fibrosis; Cloning; Expression; Osmotic water permeability; Glycerol transport; Electrolyte secretion; Ion transport; *Xenopus laevis* oocyte

## 1. Introduction

Several previous reports indicate that the cystic fibrosis transmembrane conductance regulator (CFTR) is able to interact with various membrane proteins by regulating their transport activity as well as functioning as a cAMP-regulated chloride channel [1,2]. Among these proteins, inhibition of the epithelial $Na^+$ channel (ENaC) by CFTR is of clinical relevance in cystic fibrosis (CF) and has been examined in detail [3,4]. Certain regions within the CFTR molecule that might be critical for the interaction with ENaC have been identified, including the first nucleotide binding domain (NBD1) and a C-terminal PDZ binding domain [5,6]. CFTR was also demonstrated to be associated with an enhanced osmotic water permeability [7–9]. Initially, it was claimed that a significant portion of water is conducted via the CFTR protein, once activated by cAMP dependent stimulation [10]. A subsequent study demonstrated that the osmotic water permeability that is activated through stimulation of CFTR is due to CFTR dependent activation of an independent water permeable membrane conductance in *Xenopus* oocytes [7]. This was suggested on the basis that both CFTR activated $Cl^-$ transport and water flux are either activated or inhibited independently.

The molecular nature of the protein that is responsible for these CFTR mediated increases of water permeability, however, remained obscure. Since glycerol permeability in *Xenopus* oocytes was enhanced along with the increase of osmotic water permeability, it was suggested that the enhanced water flux was due to activation of a glycerol permeable aquaporin (AQP), presumably AQP3. In accordance with that, CFTR dependent activation of osmotic water transport was demonstrated in normal human airway epithelial cells, but was not detected in airway cells derived from CF patients [8]. This study showed that 3-isobutyl-1-methylxanthine (IBMX) activated osmotic water permeability and glycerol uptake was caused by CFTR mediated activation of human AQP3. Since CFTR dependent stimulation of osmotic water permeability is absent in airway cells derived from CF patients, it was speculated that this might have a pathophysiological impact on the lung disease in CF. We now have cloned the AQP3 homologue from *Xenopus laevis* and demonstrate expression in oocytes. We further show that interaction between CFTR and xAQP3 relies on the presence of an intact NBD1.

## 2. Materials and methods

### 2.1. Cloning of a xAQP3

Total RNA was isolated from *X. laevis* A6 cells and was reverse transcribed using random hexamer primers (Superscript RT, Gibco). Degenerated oligonucleotide primers were designed that bind to highly conserved sequences around NPA motifs of the MIP protein family. Sense (5′–3′): BYNGGNCMNRTNWSNGGNGSNCA (MIPA) and CCGAATTCYTNAAYCCNGCNRTNAC (MIP1; including *Eco*RI site). Antisense (5′–3′): SBNSDNSCNARNBHNCKNGCNGG (MIPB) and CCGGATCCNARNBHNCKNGCNGGRTT (MIP2; including *Bam*HI site). Coding: B = T, G or C; D = A, T or G; H = C, T or A; K = G or T; M = A or C; N = A, T, C or G; R = A or G; S = G or C; W = A or T; Y = C or T. PCR amplification was performed using MIPA and MIPB (1 min at 94°C, 30 s at 58°C, 3 min at 72°C (five cycles) followed by 1 min at 94°C, 30 s at 42°C, 3 min at 72°C (45 cycles) and 1 min at 94°C, 30 s at 42°C, 10 min at 72°C (one cycle)). PCR products were re-amplified using MIP1 and MIP2. A 417 bp fragment was subcloned into pBluescript $SK^-$ and sequenced using a fluorescence DNA sequencer (373A, Applied Biosystem). Full length cDNA of xAQP3 was cloned using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). A library of adapter ligated, double stranded cDNA was synthesized from poly(A)$^+$ mRNA isolated from A6 cells (QuickPrep, Micro mRNA Purification Kit, Pharmacia Biotech, Sweden). RACE PCR was carried out with the gene specific (5′–3′) sense ATCCTGCTGTGACATTTGCCCTGT and antisense CAGAGTTGAAGCCCATGGAAGTTC primer. 3′-RACE (3272 bp) and 5′-RACE (661 bp) fragments were subcloned into pBluescript $SK^-$ and sequenced (EMBL Nucleotide Sequence Database, accession

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[1] The sequence of aquaporin 3 has been submitted to a public database (EMBL Nucleotide Sequence Database, accession number AJ131847).

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number AJ131847). Full length cDNA was constructed using an overlapping *Bcl*I restriction site and subcloned into an oocyte expression vector (pTLN), containing the *Xenopus* β-globin untranslated regions to boost expression in oocytes (kindly provided by Dr. T.J. Jentsch, Hamburg, Germany [11]).

*2.2. Expression of xAQP3 mRNA in Xenopus oocytes*

Poly(A)$^+$ RNA (QuickPrep, Micro mRNA Purification Kit, Pharmacia, Sweden) of *Xenopus* oocytes was reverse transcribed at 37°C for 1 h using random hexamer primers (Superscript RT, Gibco). A 382 bp xAQP3 fragment was amplified by PCR using (5′–3′) sense ATCCTGCTGTGACATTTGCC and antisense AGAGTTGAAGCCCATGGAAG primers. The PCR product was subcloned and sequenced.

*2.3. Osmotic water and glycerol permeability in Xenopus oocytes*

Isolation and microinjection of oocytes have been described in a previous report [12]. In brief, oocytes were dispersed and defolliculated by a 20 min treatment with collagenase (type A, Boehringer, Germany). Subsequently, oocytes were rinsed 10 times and kept in ND96 buffer (in mmol/l): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, pH 7.55, supplemented with theophylline (0.5 mmol/l) and gentamicin (5 mg/l) at 18°C. Oocytes of identical batches were injected with 20 ng cRNA of wild type (wt) CFTR, G551D-CFTR, xAQP3 and truncated forms of CFTR: M595-C (C-terminal half of CFTR including R-domain but not NBD1); C590X (N-terminal half of CFTR including N-terminus, the first six transmembrane helices and NBD1 but not R-domain); M394-K830 (NBD1 and R-domain); W401M-D651X (NBD1 only). Generation of CFTR fragments has been described in previous reports [6,13]. cRNA was dissolved in 50 nl double distilled water (PV830 pneumatic pico pump, WPI, Germany). To inhibit expression of endogenous xAQP3, oocytes were injected with 5 ng of antisense oligonucleotides (5′-CAAAATCCTTCTGGCTTCCC-3′). Oocytes were injected either with sense oligonucleotides for xAQP3 (5′-GGGAAGCCAGAAGGATTTTG-3′) or with double distilled water, serving as controls. The oocyte osmotic water permeability coefficient ($P_f$) was calculated from the rate of volume increase, measured by gravimetric techniques at 22°C upon exposure to hypotonic ND96 buffer [7]. Normotonic medium (214.4 mosmol/l) was prepared from ND96 buffer by substitution of 120 mmol/l mannitol for 60 mmol/l NaCl. Hypotonic condition was induced by omission of mannitol from the above normotonic medium. $P_f$ was calculated from the equation: $P_f = dV/dt \times (1/S \times \Delta\Pi)$ where $S$ is the oocyte surface area, $V$ is the volume, and $\Delta\Pi$ the osmolality gradient at time zero [14]. $dV/dt$ was calculated from the weight change after 1 min upon exposure to hypotonic buffer. $\Delta\Pi$ was expressed as water concentration and was calculated from $[(Osm_{in} - Osm_{out})/55.6]$, where 55.6 was the molar concentration of water. Glycerol permeability coefficients ($P_{gly}$, cm/s) were calculated from the initial rate of [$^{14}$C]glycerol uptake per oocyte during incubation for 2 min in ND96 solution containing 2 μCi/ml of [$^{14}$C]glycerol and 1 mmol/l glycerol using the equation: $P_{gly}$ = uptake of glycerol/$S \times$ gradient of glycerol. Oocytes were rapidly rinsed three times with ice-cold modified ND96 solution. Each oocyte was lysed overnight in 100 g/l sodium dodecyl sulfate at room temperature. Radioactivity was measured by liquid scintillation.

*2.4. Materials and statistics*

All compounds used for the experiments were of the highest grade of purity available. IBMX was from Sigma (Deisenhofen, Germany). Data are given as mean values ± S.E.M. ($n$), where $n$ refers to the number of experiments. Paired and unpaired $t$-tests were used to compare mean values within and between series, respectively. A $P$ value of ≤0.05 was accepted to indicate statistical significance.

## 3. Results

*3.1. Cloning and expression of xAQP3*

AQP3 was cloned from *Xenopus* A6 cells by RACE-PCR. The full length mRNA consists of 3514 bases and encodes a protein of 292 amino acids (EMBL Nucleotide Sequence Database, accession number AJ131847) (Fig. 1A). The xAQP3 amino acid sequence shows a high degree of identity (78%)

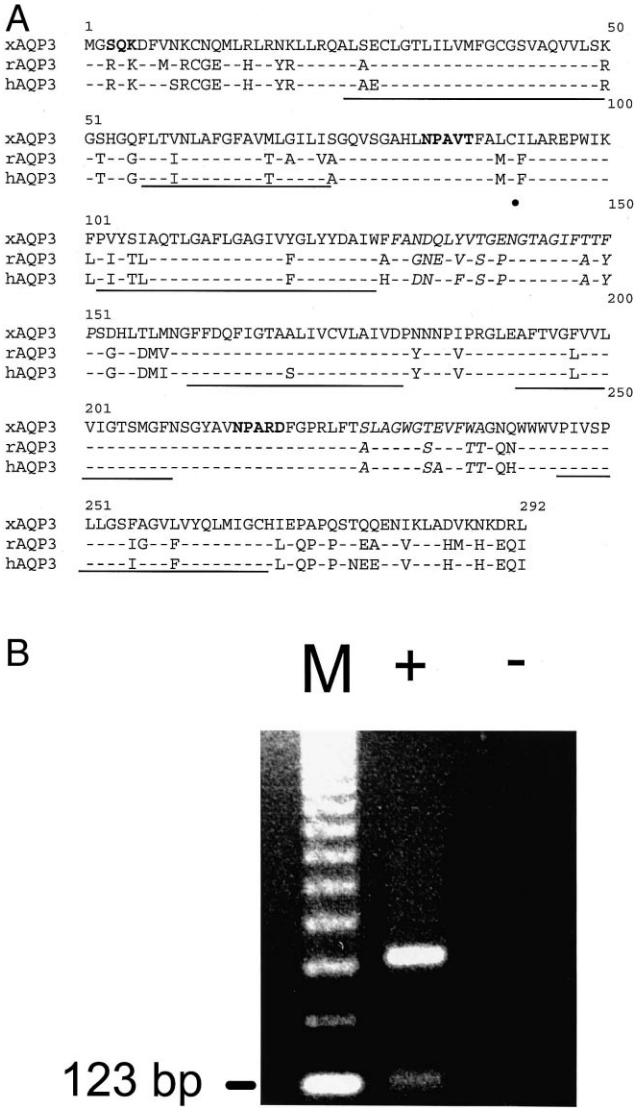


Fig. 1. A: Alignment of the amino acid sequence of xAQP3 with that of human and rat AQP3. The homologies of the amino acid sequences with human and rat AQP3 are 79% and 77%, respectively. Six potential transmembrane domains are underlined. One potential *N*-linked glycosylation site is indicated by an asterisk. The conserved NPAVT and NPARD sequences are in bold and sequences homologous to glycerol facilitator of *E. coli* are written in italics. The sequence SQK (bold) is a potential PKC phosphorylation site. B: RT-PCR analysis of the expression of xAQP3 mRNA in *Xenopus* oocytes. A specific 382 bp xAQP3 fragment was amplified after reverse transcription (RT) of mRNA (+) but not without RT (−).

when compared with corresponding sequences obtained in rat and human. xAQP3 contains NPAVT and NPARD sequences conserved among proteins of the aquaporin family and contains sequences homologous to the glycerol facilitator of *Escherichia coli* [15,16]. Reverse transcribed mRNA isolated from *Xenopus* oocytes and analyzed by RT-PCR indicated expression of xAQP3 in *Xenopus* oocytes (Fig. 1B). Oocytes were exposed to hypotonic bath solution and osmotic cell swelling was detected by gravimetric measurements as described in previous reports [7]. Overexpression of xAQP3 resulted in a significant increase of osmotic cell swelling (osmotic weight increase) and augmentation of the osmotic water permeability coefficient ($P_f$) in *Xenopus* oocytes (Fig.

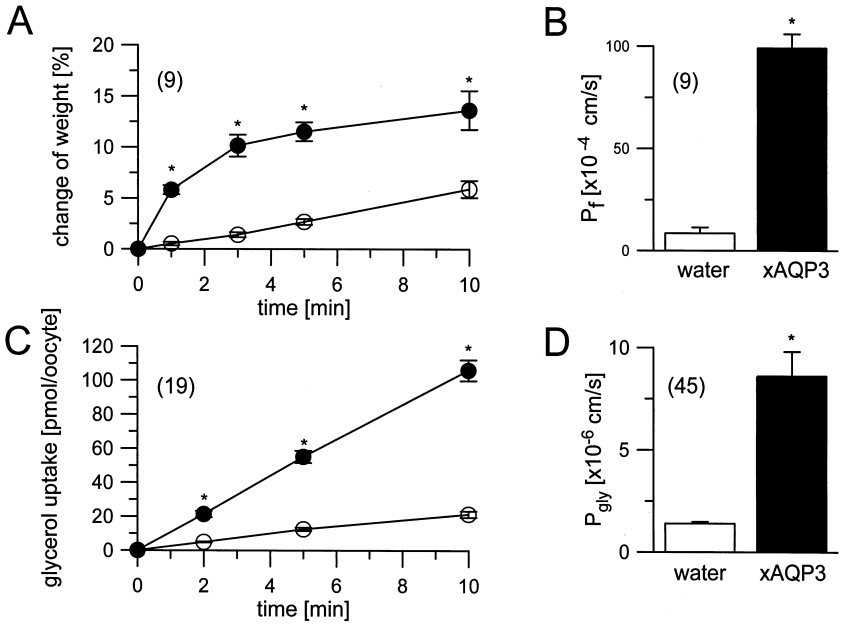


Fig. 2. Overexpression of xAQP3 increases water and glycerol permeability in *Xenopus* oocytes. A: Recording of changes in oocyte weight as a measure for cell swelling in water injected (○) and xAQP3 expressing oocytes (●). B: Osmotic water permeability coefficient ($P_f$) values calculated for water and xAQP3 injected oocytes. C: Time course of glycerol uptake in water injected (○) and xAQP3 expressing oocytes (●). D: Glycerol permeability coefficient ($P_{gly}$) calculated for water and xAQP3 injected oocytes. Asterisks indicate significant difference from control. Figures in parentheses indicate the number of experiments.

2A,B). Because cell swelling levels off after several minutes, probably due to secondary regulatory decrease, initial slopes were used to calculate $P_f$ and $P_{gly}$ values, respectively. Expression of xAQP3 was also assessed by enhanced glycerol uptake as shown in Fig. 2C. Calculation of the glycerol permeability coefficient ($P_{gly}$) indicated a large increase in $P_{gly}$ in xAQP3 overexpressing oocytes (Fig. 2D).

### 3.2. Activation of xAQP3 by CFTR

Previous reports demonstrated an increase in glycerol permeable osmotic water permeability in *Xenopus* oocytes upon expression and stimulation of the CFTR [7,9,10]. Here we demonstrate that stimulation with IBMX (1 mmol/l) leads to a significant increase in membrane conductance ($G_m$) and $P_{gly}$ in oocytes expressing wtCFTR (Fig. 3). As demonstrated in previous reports [7,8], IBMX had no effects on water injected control oocytes or AQP3 expressing oocytes (data not shown). Stimulation of oocytes expressing G551D-CFTR only slightly increases $G_m$ but does not affect $P_{gly}$. The increase of $P_{gly}$ observed in wtCFTR expressing oocytes was completely blocked by coinjection of antisense oligonucleotides for xAQP3, while injection of sense oligonucleotides had no effect (Fig. 3A). Antisense oligonucleotides for human AQP1 and AQP5 were also ineffective as demonstrated recently [8]. Both sense and antisense did not inhibit IBMX activated whole cell conductance. These results indicate that CFTR stimulated glycerol permeable water permeability is caused by activation of an independent water channel as suggested in an earlier study [7] and that this water channel is identical to AQP3 expressed endogenously in *Xenopus* oocytes.

### 3.3. NBD1 of CFTR activates xAQP3

CFTR acts as a $Cl^-$ channel and as a regulator of other membrane conductances [1,2]. It has been suggested previ-

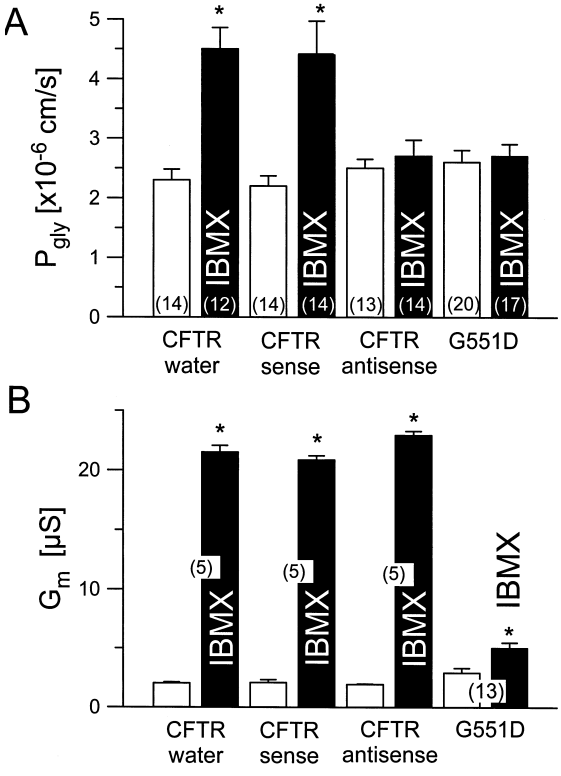


Fig. 3. Effects of antisense oligonucleotide injection on (A) glycerol permeability ($P_{gly}$) and (B) whole cell conductances ($G_m$) in *Xenopus* oocytes. Oocytes were coinjected with wtCFTR and water, sense or antisense oligonucleotides for xAQP3, or were injected with G551D-CFTR. Asterisks indicate significant difference from control. Figures in parentheses indicate the number of experiments.

ously that certain domains of CFTR, like NBD1, are essential for both activation of the outwardly rectifying $Cl^-$ channel and inhibition of the amiloride sensitive $Na^+$ conductance (ENaC) [6,17,18]. Here, we examined whether CFTR's ability to activate xAQP3 is localized in certain domains of the protein. To this end, wtCFTR and a CFTR fragment comprising NBD1 and R-domain of CFTR (M394-K830) was expressed in *Xenopus* oocytes and osmotic cell swelling was assessed in the absence or presence of IBMX (1 mmol/l). Previous studies demonstrated expression of these CFTR truncations in *Xenopus* oocytes [6,17]. As demonstrated in Fig. 4, osmotic cell swelling (as detected by change of weight) was reversible in both the absence and presence of IBMX. However, cell swelling was largely increased after stimulation with IBMX in oocytes expressing wtCFTR but not in water injected control oocytes. The same was observed for oocytes expressing the NBD1-R fragment of CFTR. This indicates that a stretch of amino acids located either in NBD1 or in the R-domain is responsible for the effects of CFTR on xAQP3.

In order to further isolate domains participating in CFTR dependent activation of xAQP3, we measured IBMX induced glycerol uptake in oocytes expressing various truncations of CFTR. As summarized in Fig. 5, we found that full length CFTR, a N-terminal half protein which lacks of the R-domain and even a CFTR fragment containing NBD1 only, are all able to activate xAQP3. The C-terminal half of CFTR including the R-domain, however, did not have any effects on $P_{gly}$. These results correspond well with others obtained for the CFTR dependent regulation of ion channels such as ENaC, ORCC and ROMK1 and indicate that an important regulatory site is located in NBD1 of CFTR [6,17,19].

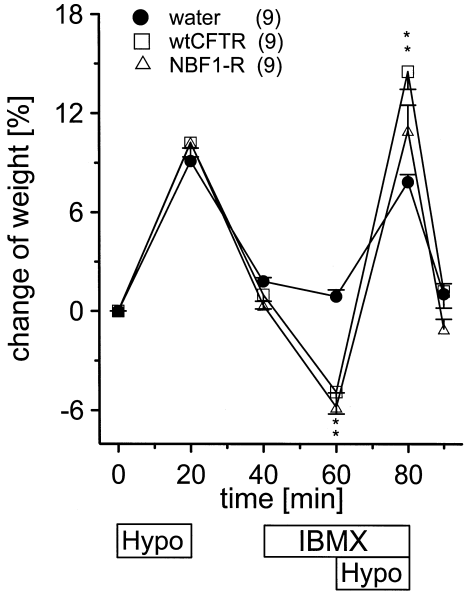


Fig. 4. Effects of stimulation with IBMX (1 mmol/l) on cell swelling (as measured by weight change) in oocytes expressing wtCFTR or a fragment comprising NBD1 and R-domain of CFTR (NBD1-R) and water injected oocytes. In water injected oocytes cell swelling and thus increase in cell weight by hypoosmotic media (Hypo, 94.4 mosmol/l) was independent of stimulation with IBMX. In oocytes expressing CFTR or NBD1-R stimulation with IBMX induced initial cell shrinkage in the presence of normotonic medium and enhanced cell swelling when bathed in hypotonic medium.

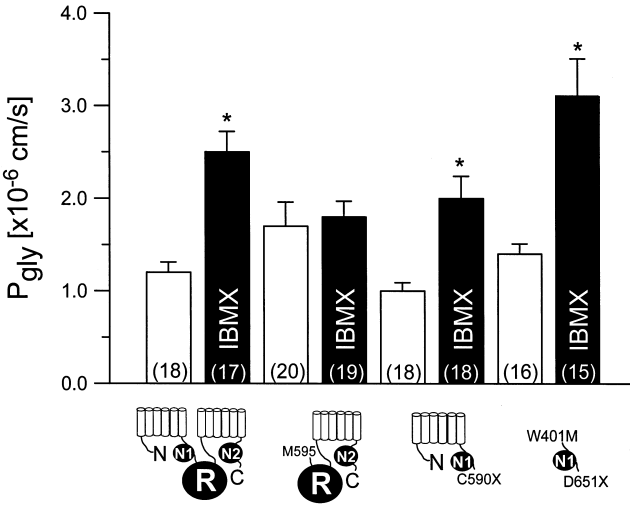


Fig. 5. Effects of the expression and stimulation by IBMX (1 mmol/l) of wtCFTR and various truncated versions of CFTR on the glycerol permeability coefficient ($P_{gly}$). After stimulation $P_{gly}$ was enhanced in wtCFTR, C590X and W410M-D651X expressing oocytes but not in oocytes injected with the C-terminal half of CFTR (M595-C). Asterisks indicate significant difference from control.

## 4. Discussion

The present data deliver new insights into the mechanism of CFTR dependent activation of endogenous water permeability in *Xenopus* oocytes. This finding was interpreted initially as water transport through the $Cl^-$ conductive pore of CFTR [10] but was shown later to be caused by CFTR dependent regulation of a glycerol permeable osmotic water permeability [7]. The present experiments now demonstrate that CFTR interacts with endogenous *Xenopus* AQP3 and xAQP3 expressed exogenously. This finding supports previous results showing CFTR dependent regulation of AQP3 in human airway epithelial cells [8]. The results suggest functional coupling between $Cl^-$ transport as performed by the CFTR $Cl^-$ channel and water transport performed by AQP3. This mechanism would help to adjust water permeability to electrolyte transport in the airway epithelium and would secure proper hydration of the airway surface fluid and mucociliary clearance [20,21].

CFTR's function as a cAMP regulated $Cl^-$ channel is well documented [9]. Recent studies demonstrate its ability to regulate other membrane conductances. CFTR has been shown to activate outwardly rectifying $Cl^-$ channels (ORCC, ICOR) upon cAMP dependent stimulation [3,18,22,23]. NBD1 and R-domain of CFTR are essential for regulation of ICOR. NBD1 of CFTR is also crucial for conferring glibenclamide sensitivity to $K_{ATP}$ channels [19,24–26]. CFTR dependent regulation of ENaC depends on the presence of $Cl^-$ ions and the existence of NBD1 [6,27]. The results reported here also emphasize the importance of NBD1 for CFTR dependent activation of AQP3. Thus, NBD1 seems to contain a motif that allows interaction with various other integral membrane proteins by either physical interaction or coupling to unidentified signal molecules [1,28]. A rather direct interaction was suggested from some studies showing inhibition of ENaC by CFTR [13,27,29,30]. The recently identified PDZ1 binding domain in the C-terminal tail of CFTR was suggested to participate in the interaction with other membrane proteins

[5,31]. However, mutating the PDZ1 binding domain did not interfere with CFTR dependent inhibition of ENaC (unpublished data). Moreover, the data shown here and elsewhere clearly indicate that NBD1 alone is capable of conferring effects on ENaC and AQP3 [6].

The NBD1-R fragment of CFTR enhanced cell swelling upon stimulation with IBMX and thus was able to activate xAQP3. It also induced cell shrinkage when applied under normotonic conditions, similar to wtCFTR. As demonstrated previously, cell shrinkage is caused by activation of a $Cl^-$ conductance and $Cl^-$ exit. In fact, a whole cell $Cl^-$ current was generated by expression of NBD1-R and stimulation by IBMX (data not shown). This suggests that either NBD1-R is able to conduct $Cl^-$ as suggested in a previous report [32], or it activates an endogenous $Cl^-$ conductance in a cAMP dependent manner. N-terminal half proteins of CFTR have been reported to function in a similar way [1,6,33].

Regulation of membrane conductances by CFTR is not limited to cells expressing recombinant proteins but can be detected in cells expressing the respective proteins endogenously, such as native respiratory and colonic epithelial cells [8,34,35]. How the interaction between CFTR and AQP3 takes place in the intact tissue is currently under investigation. In case of AQP3 as the interacting protein, the situation is somehow complicated by the fact that in the airways CFTR is expressed predominantly in apical membranes of submucosal gland cells and surface epithelial cells, while AQP3 is located in basolateral membranes. The same applies to expression of AQP3 in the gastrointestinal tract [36–38]. Thus, both CFTR and AQP3 are expressed on different poles in airway and intestinal cells. Generation of a soluble signal molecule would probably be required to facilitate interaction of AQP3 and CFTR in polarized epithelial cells. GTP binding proteins might participate in the regulation of ICOR by CFTR [39] and could also play a role in CFTR dependent regulation of AQP3 [40].

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