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Aquaporin 3, a glycerol and water transporter, is regulated by p73 of the p53 family

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Abstract p73, a member of the p53 family, has been shown to exhibit similar biochemical activities to that of p53. However, in contrast to p53, p73 is rarely mutated in human tumors and p73 mutant mice develop neurological, pheromonal, and inflammatory defects, but not spontaneous tumors. Furthermore, p73 mutant mice are deficient in the physiological control of cerebral spinal fluid. To determine what mediates these p73 activities, cDNA subtraction assay was performed to identify cellular genes that are regulated by p73. We found that aquaporin 3 (AQP3), a glycerol and water transporter, is regulated by p73. In addition, we identified a potential p53 response element in the promoter of the AQP3 gene, which is responsive to p73. This suggests that AQP3 may mediate the activity of p73 in maintaining cerebral spinal fluid dynamics.

Key words: p73; p53; AQP3; Glycerol; Water transporter

1. Introduction

p73, a member of the p53 family, shares a considerable sequence similarity with p53, especially in the transactivation, DNA binding, and tetramerization domains [1]. p73 is expressed as at least six different splicing variants: p73α, p73β, p73γ, p73δ, p73ε, and p73ζ [2,3]. A ΔN variant of these p73 proteins has been found, which lacks the N-terminal activation domain and is potentially dominant negative [4]. Like p53, p73 can bind to the p53 response element and regulate some p53 target genes [2,3]. p73 can also induce cell cycle arrest and apoptosis [2,3]. However, unlike p53, p73 is not frequently mutated and p73 mutant mice are not susceptible to spontaneous tumors [2–4]. Instead, p73 is necessary for the normal neurological and pheromonal development and the inflammatory response [4]. Mutant p73 mice are also abnormal in maintaining spinal fluid dynamics [4].

The aquaporins are a family of small transmembrane water and/or glycerol transporters. Currently, ten aquaporins (AQP0–AQP9) have been identified and are divided into two groups, 'aquaporins' and 'aquaglyceroporins'. The aquaporin group, including AQP0, 1, 2, 4, 5, 6, and 8, is structurally related to the bacterial water channel and highly selective for the passage of water [5–8]. The aquaglyceroporin group, including AQP3, 7, and 9, is structurally related to the bacterial glycerol facilitator with an enlarged loop E and permeated by water, glycerol, and some other solutes [5–8]. AQP3 is relative weak in the transport of water, but very efficient in the transport of glycerol and other solutes [9–12]. AQP3 is initially cloned from the kidney collecting duct and brain meningeal cells [9,10,13] and later found to be expressed in red and dendritic cells and epithelium cells from a variety of tissues, including tracheal, eye, nasopharyngeal, digestive tract, and skin [14–18]. Like AQP2 mutant mice, mice deficient in AQP3 are susceptible to nephrogenic diabetes insipidus but developmentally normal [19]. In an effort to identify cellular genes that mediate p73 activity, we performed cDNA subtraction assay and found that AQP3 is regulated by p73.

2. Materials and methods

2.1. Cell culture and cell lines

MCF7 and H1299 cell lines were purchased from American Type Culture Collection (Rockville, MD, USA). p53-24, p53(R249S)-25, p73α-2, p73α292-3 and p73β-38, derivatives of the MCF7 cell line that inducibly express wild-type p53, p53(R249S), p73α, p73α292, and p73β, respectively, were generated as previously reported [20].

2.2. RNA isolation, cDNA subtraction assay, and Northern blot analysis

Poly(A)+ RNA was isolated from p73β-38 cells using an mRNA purification kit (Pharmacia, Piscataway, NJ, USA). Total RNA was isolated from cells using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD, USA). The cDNA subtraction assay was performed using the Clontech PCR-select cDNA subtraction kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). Subtracted cDNA fragments were cloned into pGEM-T vector (Promega, Madison, WI, USA). Northern blot analysis was performed as described previously [20]. p21 and GAPDH probes were prepared as described previously [20]. A 615-bp *Not*I fragment, prepared from AQP3 cDNA [21], was used to detect AQP3.

2.3. Luciferase assay

A 36-bp fragment (5′-AAGCTAGGTCACCAGCCATGTTCAACAGGCATGTGC-3′) that contains the potential p53 response element in the AQP3 promoter was synthesized and cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene [22]. The resulting construct was designated AQP3-Fluc. A 990-bp fragment that contains the potential p53 response element and a 790-bp fragment that lacks the potential p53 response element in the AQP3 promoter were generated by PCR and cloned into a promoterless luciferase reporter vector (pGL2-basic) (Promega, Madison, WI, USA). The resulting constructs were designated APP990 and APP790. 2 μg of AQP3-Fluc, APP990, or APP790 was cotransfected into H1299 cells with 1 μg pcDNA3 control vector or a vector that expresses p53, p53(R249S), p73α, p73α292, p73β, or p73β292. 0.1 μg of renilla luciferase assay vector, pRL-CMV (Promega), was also cotransfected as an internal control. The dual luciferase assay was performed according to the manufacturer's instructions (Promega).

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2.4. Immunocytochemistry

Cells were plated on four-chamber tissue culture slides. After washing with PBS, the cells were fixed with 10% formalin, permeabilized with 1% NP-40, and incubated in 15% goat or rat serum to block nonspecific binding. The cells were then stained with anti-p73 (Ab-2, Oncogene, Cambridge, MA, USA) or anti-AQP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody and examined by fluorescence microscope.

3. Results

To identify novel target genes regulated by p73, the Clontech PCR-select cDNA subtraction assay was performed using mRNA isolated from the p73β-38 cell line, a derivative of the MCF7 cell line that inducibly expresses p73β under the control of a tetracycline-regulated promoter. Several cDNA fragments that may represent genes induced by p73 were isolated. After DNA sequencing, one subtracted cDNA fragment was found to be derived from the aquaporin (AQP) 3 gene. To confirm that AQP3 can be induced by p73, Northern blot analysis was performed using AQP3 cDNA as probe. We found that AQP3 was strongly induced in p73β-38 cells when p73β was expressed (Fig. 1, AQP3 panel, compare lanes 5 and 6). AQP3 was also induced by p73α (Fig. 1, AQP3 panel, compare lanes 1 and 2) and weakly induced by p53 (Fig. 1, AQP3 panel, compare lanes 7 and 8). In contrast, mutant p73α292 and p53(R249S) were incapable of inducing AQP3 (Fig. 1, AQP3 panel, compare lanes 3, 4 and 9, 10). As a control, we tested the expression of p21, a well-defined target gene for both p53 and p73. We found that p21 was induced by p53, p73α, p73β (Fig. 1, p21 panel), but not by mutant p53(R249S) and p73α292 (Fig. 1, p21 panel).

To determine whether AQP3 is transcriptionally regulated by p73, we searched for a potential p53 response element that can be regulated by p73. To do this, we screened a human bacterial artificial chromosome (BAC) library (The Genome System, St. Louis, MO, USA) and identified a clone that contains the entire AQP3 locus. A region of 3570 nucleotides in the promoter of the AQP3 gene was sequenced (data not shown). We found one potential p53 response element (Fig. 2A). This sequence (AAG CTAG ***g***TC acc AG***c*** CATG TTC aac AGG CATG T***g***C) contains three half sites with only one mismatch each (in bold italics) in the non-critical region as compared to the consensus p53 response element [23,24].

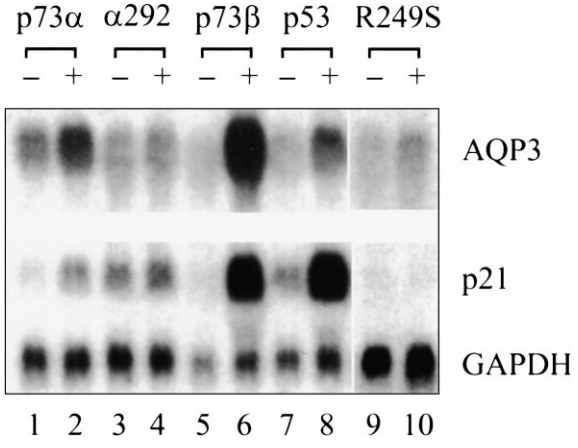

Fig. 1. Upregulation of AQP3 by p73. Northern blots were prepared using 10 μg of total RNA isolated from p73α-2, p73α292-3, p73β-38, p53-24, or p53(R249S)-25 cells under both the uninduced (−) and induced (+) conditions. The blots were probed with cDNAs derived from the AQP3, p21, and GAPDH genes, respectively.

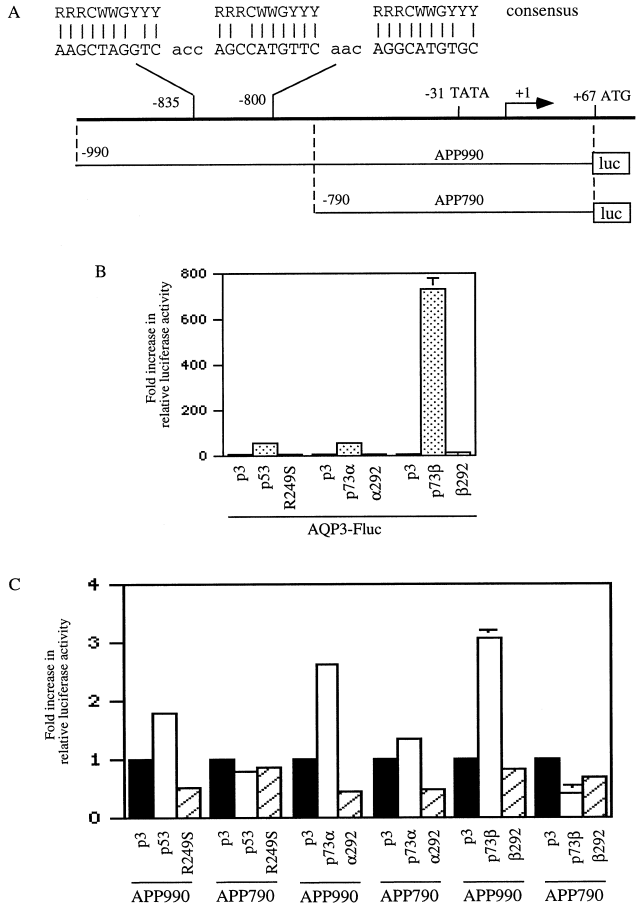

Fig. 2. Identification of a potential p53 response element in the AQP3 gene. A: Schematic representation of the AQP3 genomic structure. The position of the AQP3 transcription and translation start sites and a potential p53 response element are indicated. Shown above the genomic structure are the sequence of the potential p53 response element and the previously characterized consensus response element. R represents purine, Y pyrimidine, and W adenine or thymidine. Shown below the genomic structure is the location of the DNA fragments used to generate two reporter constructs. B: The potential p53 binding site in the AQP3 gene is responsive to p53 and p73, but not to mutant p53 and p73. 2 μg of AQP3-Fluc was cotransfected into H1299 cells with 1 μg of pcDNA3 control vector or a vector that expresses p53, p53(R249S), p73α, p73α292, p73β, or p73β292. The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 or p73 divided by that induced by pcDNA3. C: The AQP3 promoter that contains the potential p53 binding site is responsive to p53 and p73, but not to mutant p53 and p73. The experiment was performed as in B.

To analyze whether this element is responsive to p53 and p73, we cloned a 36-bp fragment containing the potential p53 response element upstream of the c-*fos* minimal promoter and a luciferase reporter gene [22] to generate a reporter vector, designated AQP3-Fluc. AQP3-Fluc was cotransfected into H1299 cells with either a pcDNA3 control vector or a vector expressing p53, p53(R249S), p73α, p73α292, p73β, or p73β292. We found that the luciferase activity for AQP3-Fluc was markedly increased by p53 (52-fold), p73α (52-fold), and p73β (731-fold), but not by mutant p53(R249S), p73α292, and p73β292 (Fig. 2B).

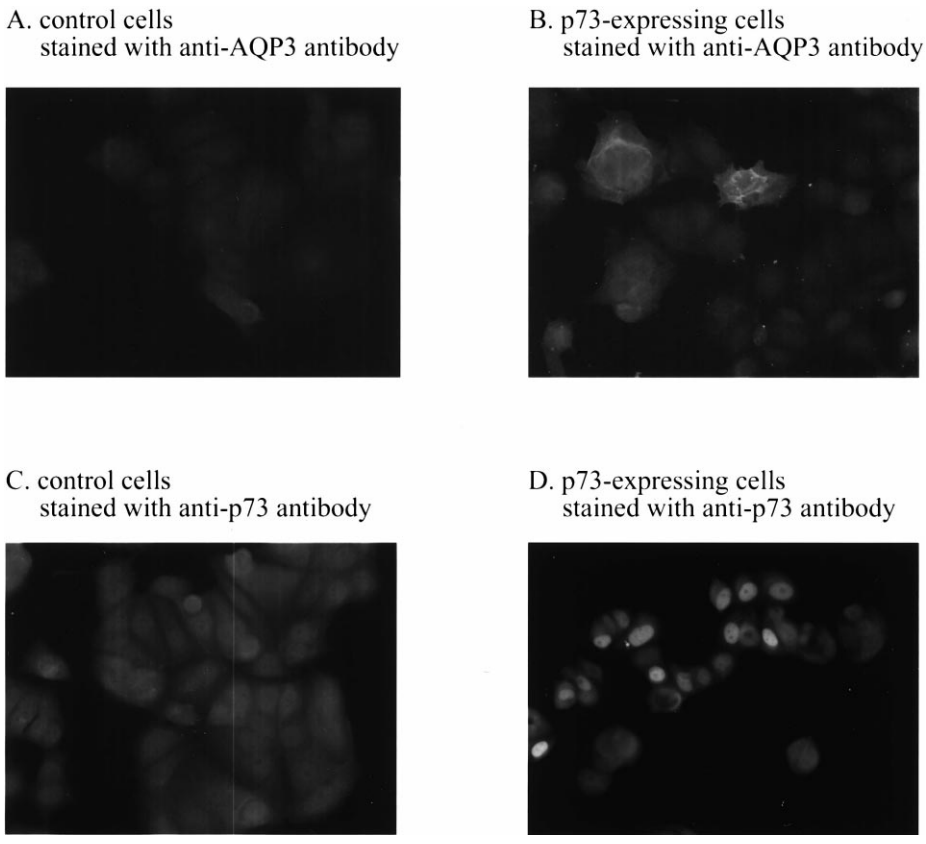

Fig. 3. Immunological detection of the AQP3 protein in p73-expressing cells. p73β-38 cells were uninduced or induced for 24 h, fixed in 4% formalin, and stained with anti-AQP3 or anti-p73 antibody. A: Control cells stained with anti-AQP3 antibody. B: p73β-expressing cells stained with anti-AQP3 antibody. C: Control cells stained with anti-p73 antibody. D: p73β-expressing cells stained with anti-p73 antibody.

To determine whether p53 and p73 can regulate the potential p53 response element in the natural promoter of the AQP3 gene, we cloned the promoter region of the AQP3 gene into a promoterless luciferase reporter vector (pGL2-basic). The resulting vectors are designated APP990 and APP790 (Fig. 2A). APP990, but not APP790, contains the potential p53 response element. We found that the luciferase activity for APP990, but not for APP790, was increased by p73α (2.6-fold), p73β (3.1-fold), and p53 (1.7-fold) (Fig. 2C). In contrast, mutant p53(R249S), p73α292, and p73β292 were incapable of increasing the luciferase activity for both APP990 and APP790. It should be mentioned that the ability of p73β to increase the luciferase activity for both AQP3-Fluc and APP990 was much higher than that by p53 and p73α, consistent with the strong induction of endogenous AQP3 by p73β (Fig. 1).

To determine whether the enhanced expression of the AQP3 gene leads to an increased expression of the AQP3 protein in p73β-expressing cells, we measured the level of the AQP3 protein in cells by immunofluorescence microscopy. We found that the level of the AQP3 protein was substantially increased in p73β-expressing cells as compared to control cells (Fig. 3, compare A and B). We also found that the AQP3 protein was localized on the inner plasma membrane (Fig. 3B), as previously reported [17,25,26]. As a control, we found that p73β was expressed in the nucleus only when induced (Fig. 3, compare C and D).

4. Discussion

Several studies have shown that AQP3 can be regulated by the corticosteroid dexamethasone, activated protein kinase C, and cystic fibrosis transmembrane conductance regulator in airway epithelial cells and lung carcinoma A549 cells [21,26,27]. However, the mechanism by which AQP3 is regulated is still not clear. In this study, we found that AQP3 is strongly induced by p73 and weakly induced by p53. In addition, we have identified a potential p53 response element in the promoter of the AQP3 gene, which is responsive to p73 and p53.

Although p73 and p53 are highly similar in the DNA binding domain, we and others have shown that p73 and p53 differentially regulate p53 target genes [28–30]. For example, 14-3-3σ is strongly induced by p73 whereas p21 and MDM2 are strongly induced by p53. Thus, AQP3 becomes another example. Since the cellular target genes are responsible for the activities of the p53 family members, the differential regulation of cellular genes by the p53 family members may correlate with the distinct activities of p53 and p73 [2,3]. Therefore, AQP3, as a p73 target, may mediate the activity of p73 in regulating the homeostasis of cerebral spinal fluid. In mice, p73 is highly expressed in the epithelial cells of the choroid plexus and the ependymal cells lining the ventricles [4], both of which participate in regulating cerebral spinal fluid dynamics. Therefore, lack of p73 leads to defects in production or reabsorption of cerebral spinal fluid, resulting in hydrocepha-

lus [4]. Among the aquaporin family, AQP3 and AQP4 are expressed in the brain ependymal cells [10,14]. Since AQP4 is not induced by p73 (data not shown), further studies are needed to determine whether lack of AQP3 induction by p73 contributes to the hydrocephalus in the p73 mutant mice.

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