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A novel 14-3-3 gene is osmoregulated in gill epithelium of the euryhaline teleost *Fundulus heteroclitus*

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Summary

We have cloned and analyzed the full-length coding sequence and 3′ untranslated region (UTR) of a unique 14-3-3 gene of the euryhaline teleost *Fundulus heteroclitus*, which we named 14-3-3.a. Phylogenetic analysis of the deduced amino acid sequence revealed that the 14-3-3.a gene product is most similar to vertebrate 14-3-3ζ and β, yet it displays considerable divergence to known classes of vertebrate 14-3-3 isoforms. The N and C termini of 14-3-3.a are the most unique regions, whereas the amino acid residues forming the amphipathic ligand-binding groove are highly conserved. *F. heteroclitus* 14-3-3.a mRNA expression is high in gill epithelium, moderate in intestine and brain, and low in gonads, white muscle and heart. Because 14-3-3 proteins are important molecular scaffolds and cofactors for phosphoproteins and signaling complexes, the high level of 14-3-3.a expression in gill epithelium of the euryhaline teleost *F. heteroclitus* suggests that it is crucial for signal transduction in gill epithelial cells. We provide evidence that 14-3-3.a is involved in osmosensory signal transduction by showing that its mRNA and protein levels in gill epithelium, but not in any other tissue analyzed, increase two- to fourfold within 24 h of salinity transfer of fish from sea water to fresh water. These data are clear evidence for an important role of 14-3-3.a in the remodeling of gill epithelium during transition of euryhaline fish between plasma-hyperosmotic and plasma-hyposmotic environments.

Key words: osmoregulation, signal transduction, 14-3-3, gene expression, gene regulation, protein phosphorylation, euryhaline fish, gill epithelium, salinity adaptation, chloride cell, killifish, *Fundulus heteroclitus*.

Introduction

Euryhaline teleosts maintain their plasma osmotic homeostasis by extrarenal transport of salt. In a plasma-hyperosmotic environment (e.g. sea water, SW) they actively secrete NaCl, while in a plasma-hyposmotic environment (e.g. fresh water, FW) NaCl is actively absorbed across the gill epithelium. Accordingly, the cellular organization of the gill epithelium of euryhaline fishes differs largely between FW and SW (Laurent and Dunel, 1980). During acclimation of euryhaline fish from SW to FW and *vice versa* the gill epithelium is extensively remodeled to account for the altered requirements of ion transport. Such remodeling includes changes in the turnover of gill epithelial cells (Conte and Lin, 1967; Chretien and Pisam, 1986; Uchida and Kaneko, 1996), altered differentiation patterns of gill epithelial cells (Conte and Morita, 1968; Kültz et al., 1995), and the modulation of the activity of ATPases and ion transporters (Evans, 1993; Kültz and Somero, 1995). 14-3-3 proteins are excellent candidates for molecules that control the reorganization of gill epithelium during salinity adaptation of euryhaline teleosts because they are ubiquitous phosphoprotein partners and very potent master regulators that control the activity of many signal transduction pathways in response to a changing environment (Fu et al., 2000). Interestingly, many molecular phenomena involved in salinity adaptation of euryhaline fish are features that are regulated by 14-3-3. Examples are the activation of H^+-ATPase, the regulation of cell proliferation and turnover, the regulation of apoptosis, protein kinase C (PKC) regulation that is important for the modulation of Na^+/K^+-ATPase activity, the regulation of ion channels and transporters, and the regulation of the cytoskeleton. 14-3-3 proteins have in common that they are essential for the adaptation of virtually all eukaryotic cells to a changing environment, which is reflected by a remarkably high degree of structural conservation of 14-3-3 proteins in all eukaryotes (Finnie et al., 1999). A striking general property of 14-3-3 proteins concerns their binding and regulation of a very large number of protein kinases, phosphatases and other phosphoproteins (Fu et al., 2000). The wide variety of 14-3-3 partners resembles the large number of calmodulin targets and suggests that 14-3-3 proteins are ubiquitous regulators of cellular physiology and biochemistry. It is now recognized that 14-3-3 proteins are important convergence points of many, if not all, important signal transduction pathways (Fu et al., 2000). In addition, one specific 14-3-3 isoform, 14-3-3ζ, has been identified as an

arachidonate-selective acyltransferase and phospholipase A2 (PLA2) (Luo et al., 1995; Du et al., 1994) and 14-3-3 proteins are important for vesicle trafficking and Ca^{2+}-dependent exocytosis (Roth et al., 1999; Chamberlain et al., 1995). These properties of 14-3-3 suggest that they are important for osmosensory signal transduction during hyposmotic stress (SW to FW transition) because PLA2 is a potential primary osmosensor and Ca^{2+}-dependent exocytosis is a hallmark for cellular adaptation during hyposmotic stress (Lehtonen and Kinnunen, 1995; Hoffmann and Pederson, 1998; Kinne, 1998). In this study we have cloned the first 14-3-3 gene from a euryhaline fish, analyzed its molecular structure, measured the expression in gill epithelial cells compared to other tissues, and investigated the osmotic regulation of this gene *in vivo* during osmotic challenge of fish.

Materials and methods

Animals and acclimation procedures

The euryhaline killifish, *Fundulus heteroclitus*, was used for all experiments and as the source for cloning procedures. RNA used for cloning was isolated from gill epithelium (scraped from the cartilage) of fish collected in seawater habitat near Mount Desert Island, ME, USA in the summer of 1999. Before collection of gill tissue, blood was removed from gill vasculature by brief (1–2 min) perfusion with Hank's buffered saline solution (HBSS) *via* the *bulbus arteriosus*. Salinity acclimation experiments were performed in the summer of 2000 with fish collected again in seawater habitat near Mount Desert Island, ME, USA. These fish were kept in running-seawater tanks (950 mosmol kg^{-1} H_2O) and fed *ad libitum* with commercially available trout pellets for 15 days. Following this pre-acclimation, the animals were divided into three groups: one group was left in SW (950 mosmol kg^{-1} H_2O). A second group was transferred abruptly to FW (approx. 50 mosmol kg^{-1} H_2O), and a third group was abruptly transferred to double-strength SW (1900 mosmol kg^{-1} H_2O). All tanks were vigorously aerated, the water was cycled across external charcoal filters, and animals were not fed during the acclimation period. There was no mortality and no visible discomfort of the animals following the salinity transfer. After 24 h acclimation to these three different salinities fish of all groups were sacrificed by spinal cord transection in the back of the neck. Blood was removed from gill vasculature by perfusion with HBSS via the *bulbus arteriosus* and total RNA of gill epithelium (scraped from the cartilage), brain, intestine, white muscle, liver and gonads was immediately isolated as described below. The salinity of the acclimation water was measured using a vapor pressure osmometer (Wescor) calibrated with H_2O standards (100, 1000 and 2000 mosmol kg^{-1}) prior to measurement of samples. Double-strength SW was prepared by addition of an appropriate amount of NaCl to regular SW.

Cloning, PCR and sequencing procedures

Total RNA was isolated using the phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was obtained from total RNA by affinity purification with Oligotex magnetic beads (Qiagen). A SMART cDNA library was constructed from gill epithelial poly(A)$^+$ RNA using reagents from Clontech, and Superscript II reverse transcriptase (Life Technologies). This cDNA was used for reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the central region of *F. heteroclitus* 14-3-3 using a pair of degenerate primers. These primers (sense: GTNGCNTAYAARAAYGT, antisense: CCAVARNGTVA-RRTTRTC) were designed by sequence alignment of 25 vertebrate 14-3-3 cDNAs (Clustal X; Jeanmougin et al., 1998) and identification of the two most highly conserved regions. RT-PCR was performed using Advantage 2 high fidelity PCR reagents (Clontech) and the PCR product was cloned into pTAdv vector. This construct was propagated in JM109 cells (Clontech), and a single subclone was expanded and sequenced using ABI Prism BigDye 2.0 cycle sequencing reagents and an ABI Prism 310 automated DNA sequencer (PE Biosystems). After the sequence was evaluated and confirmed to correspond to a 14-3-3 gene, the 5′ and 3′ ends of the corresponding mRNA were cloned by rapid amplification of cDNA ends (RACE) PCR using one gene-specific primer and one adapter primer corresponding to the 5′ or 3′ SMART adapter of the cDNA library. RACE PCR products were cloned into pGEMT vector and sequenced as described above. A continuous full-length cDNA clone was obtained by PCR with gene-specific primers corresponding to the 5′ and 3′ ends of the 14-3-3.a mRNA fragments identified previously.

Phylogenetic and structural analysis

The primary structure of the 14-3-3.a coding sequence and deduced amino acid sequence was analyzed with the computer programs Vector NTI and AlignX (Informax, Inc.). Multiple sequence alignments and phylogenetic tree construction were carried out using Clustal X (Jeanmougin et al., 1998) and TreeView (Page, 1996) software. The primary structure of the 3′ UTR was analyzed for the occurrence of protein-binding motifs using UTRScan and UTRdb programs (Pesole and Liuni, 1999). Modeling of the three-dimensional structure of 14-3-3.a was done with the program SwissModel (Peitsch, 1996) using the crystal structures of *Homo sapiens* 14-3-3ζ (PDB codes 1QJA, 1QJB; Rittinger et al., 1999) and *Bos taurus* 14-3-3ζ (PDB codes 1A37, 1A38, 1A40; Liu et al., 1995) as templates. The structural coordinates of 14-3-3.a that were calculated during the modeling process were then visualized using SwissPDB Viewer (Guex, 1996) and WebLab Viewer (Molecular Simulations, Inc.) software.

Northern blot analysis

Total RNA (7 μg) from each sample was denatured, loaded onto 1 % agarose/formaldehyde gels and electrophoresed using a Tris-buffer system as described previously (Farrell, 1998). Subsequently, gels were briefly stained with ethidium bromide and the two ribosomal RNA bands of each sample were quantified with a Fluor-S MultiImager (Biorad). RNA was

transferred from gels to nylon membrane (Biodyne B, LifeTechnologies) by electroblotting and UV-crosslinked to the membrane in a Stratalinker (Stratagene). Riboprobes were synthesized by *in vitro* transcription of the full-length 14-3-3.a coding sequence (CDS) in the antisense direction using T7 polymerase (Ambion) and [γ-^{32}P]dCTP (NEN). Pre-hybridization and hybridization procedures were done according to the method of Farrell (Farrell, 1998) and the intensity of the 14-3-3.a mRNA bands from each sample quantified with a PhosphorImager SI (Molecular Dynamics).

Western blot analysis

The protein abundance of *F. heteroclitus* 14-3-3.a protein was investigated in gill epithelium of fish acclimated to SW or transferred for 24 or 48 h from SW to FW. We have optimized a western detection procedure for 14-3-3.a using a monoclonal antibody. Proteins were extracted from gill epithelial tissue into lysis buffer (Kültz et al., 1997), homogenized on ice using a glass–teflon homogenizer (Wheaton), and centrifuged for 10 min at 20,000 ***g*** and 4 °C. Supernatants containing 15 μg protein per sample as determined with the bicinchoninic acid assay (Pierce) were loaded on 10 % sodium dodecylsulphate-polyacrylamide gels and electrophoresed in a Protean MiniCell (Biorad) using a Tris-glycine buffer system. Gels were electroblotted as previously described (Kültz et al., 1997) and blots were blocked with 5 % bovine serum albumin for 1 h. Even loading of samples was verified by staining gels with Coomassie Blue after blotting and quantifying several high molecular mass bands that remained after electroblotting with a FluorS MultiImager (Biorad). Post-blotting staining of gels also allowed us to verify that all protein in the molecular mass range from 10 to 50 kDa was transferred from gels to blots. Blocking of blots was followed by incubation with primary antibody that was made against a synthetic polypeptide, which is 100 % identical to the N-terminal region of 14-3-3.a (catalogue number sc-1657, Santa Cruz Biotechnology, 1:1000 dilution). Then the blot was washed three times for 5 min each using Tris-buffered saline (TBS), exposed to horseradish peroxidase-labeled secondary antibody (rabbit anti-mouse IgG, NEB, 1:2000 dilution) for 1 h, washed again three times with TBS, and developed with Supersignal reagent (Pierce). Imaging and quantification of 14-3-3 bands on the blot was done with a FluorS MultiImager (Biorad).

Statistical analysis

All quantitative data shown are means ± S.E.M. of multiple samples (N=4, except for mRNA levels in gonads where N=2). Statistics software (StatMost32, DataMost Corp.) was used to test whether differences between means of different treatment groups were significant. The F-test was used for assessing whether standard deviations of two treatment groups were significantly different. Depending on its result, the F-test was followed by either a paired t-test or the Mann–Whitney test. The significance threshold was set at P<0.05 for all tests.

Results

Primary structure of 14-3-3.a

We have cloned and sequenced a novel cDNA from the euryhaline fish *F. heteroclitus* (GB AF302039). This cDNA had a total of 1659 base pairs. 34 of these nucleotide base pairs are 5′ UTR, while the CDS, including stop codon, is 744 nucleotides long, and the 3′ UTR is 881 nucleotide pairs long (Fig. 1). The CDS encodes a novel 14-3-3 protein that consists of 247 amino acid residues, has a theoretical molecular mass of 28,015 kDa and a calculated isoelectric point of 4.73 (Fig. 1). The 3′ UTR of *F. heteroclitus* 14-3-3.a is similar to that of 14-3-3ζ but does not have a high degree of similarity to known 3′ UTRs of other 14-3-3 isoforms. The similarity of the 3′ UTR to that of 14-3-3ζ is seen in both the length and the identity of the nucleotide sequence. *F. heteroclitus* 14-3-3.a 3′ UTR, excluding the poly(A)$^+$ tail, is 855 nucleotides long compared to 905 nucleotides in frog (Miura et al., 1997) and

Fig. 1. Nucleotide (black) and deduced amino acid (red) sequences of the 14-3-3.a gene from the euryhaline teleost *Fundulus heteroclitus.* The nucleotide sequence is numbered on the left side. The partial 5′ UTR (34 nucleotides), the complete CDS (741 nucleotides plus 3 nucleotides for the stop codon corresponding to 247 amino acid residues), and the complete 3′ UTR (881 nucleotides) are shown.

918 nucleotides in human (Zupan et al., 1992). The difference from frog and human sequences is mainly the lack of a continuous stretch of 40 nucleotides immediately following the stop codon (Fig. 2). The significance of this region for mRNA processing/stability is not known. In addition to similar length, the *F. heteroclitus* 14-3-3.a 3′ UTR has 47 % identical nucleotides compared to human and also to frog (Fig. 2). This 47 % degree of identity is substantially lower than that between the 3′ UTRs of human and frog (68 %). The unusual length of the *F. heteroclitus* 14-3-3.a 3′ UTR and its relatively high degree of conservation over a long period of evolutionary divergence strongly suggest that it serves for post-transcriptional regulation of this gene, possibly by modulation of mRNA stability or through direct regulation of translational efficiency.

Phylogenetic analysis of the 14-3-3.a sequence

Multiple sequence alignment of 23 vertebrate 14-3-3 proteins and the deduced amino acid sequence of our novel *F. heteroclitus* 14-3-3 protein, followed by construction of a radial phylogenetic tree, suggests that *F. heteroclitus* 14-3-3 protein may not belong to any of the already established isoforms of vertebrate 14-3-3 proteins (Fig. 3). This result was consistently obtained despite testing different alignment parameters (scoring matrix, gap penalties) and tree construction procedures (Kimura's correction, tree display method). Therefore, we named this novel fish 14-3-3 protein 14-3-3.a to indicate that it is unique in comparison to already known vertebrate 14-3-3 isoforms. The uniqueness of fish 14-3-3 primary structure may not be too surprising considering that fish are lower vertebrates and that invertebrates also have 14-3-3 isoforms that are different from those of mammals and other higher vertebrates. Of possible importance for the regulation of its activity, *F. heteroclitus* 14-3-3.a has a unique protein kinase C and casein kinase II phosphorylation site in the N terminus that is not present in other 14-3-3 proteins (Fig. 4). In addition, several other sites that may be targets of post-translational modification are present in *F. heteroclitus* 14-3-3.a (Fig. 4). The overall nucleotide sequence identity (CDS and UTRs) of the *F. heteroclitus* cDNA is higher when compared with 14-3-3ζ than with other 14-3-3 isoforms: 57 % identity to *Homo sapiens* 14-3-3ζ (Zupan et al., 1992) and 55 % identity to *Rana rugosa* 14-3-3ζ (Miura et al., 1997). Nevertheless, the nucleotide sequence identity is substantially higher between two (human and frog) 14-3-3ζ isoforms (72 %). The degree of sequence identity between *F. heteroclitus* 14-3-3.a and other vertebrate 14-3-3 proteins is much higher in the CDS than in the UTRs. At the nucleotide level, the degree of identity of the *F. heteroclitus* 14-3-3.a CDS is 72/66/62/68/66/69/73 % to 14-3-3β/γ/ε/η/σ/τ/ζ of *Mus*

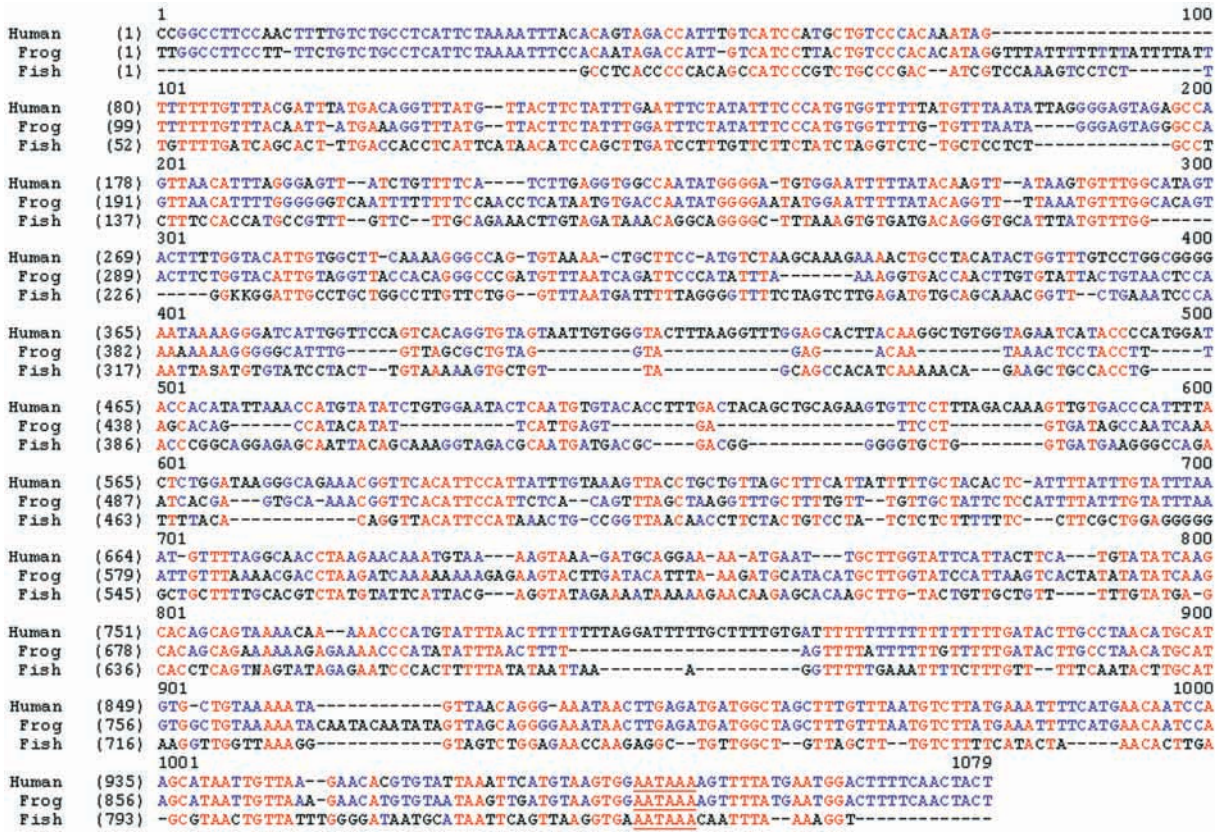

Fig. 2. Alignment of the 3′ UTR nucleotide sequences of *Fundulus heteroclitus* 14-3-3.a (Fish), *Rana rugosa* 14-3-3ζ (Frog), and *Homo sapiens* 14-3-3ζ (Human) mRNAs. Nucleotides in red are identical in all three sequences. Nucleotides in blue are identical only in two of the sequences. The polyadenylation signal is underlined in all three sequences. Nucleotide sequences are numbered on the left.

musculus, 71 % to 14-3-3ζ of *Rana rugosa*, and 63/73 % to 14-3-3ε/ζ of *Xenopus laevis*, respectively. Despite the fact that *F. heteroclitus* 14-3-3.a is most identical to 14-3-3ζ proteins, the degree of identity between two 14-3-3ζ CDSs is substantially higher (*M. musculus/R. rugosa*: 81 %, *M. musculus/X. laevis*: 83 %, *R. rugosa/X. laevis*: 79 %). This is also true at the level of amino acid sequence identity (*F. heteroclitus* 14-3-3.a/*M. musculus* 14-3-3ζ: 79 %, *F. heteroclitus* 14-3-3.a/*R. rugosa* 14-3-3ζ: 78 %, *F. heteroclitus* 14-3-3.a/*X. laevis*: 78 %, *M. musculus/R. rugosa*: 92 %, *M. musculus/X. laevis*: 90 %, *R. rugosa/X. laevis*: 87 %). Thus, even though all mouse 14-3-3 isoforms have regions of high identity with *F. heteroclitus* 14-3-3.a (Fig. 4) and the amino acid sequence of the *F. heteroclitus* protein is most similar to 14-3-3ζ proteins, this novel fish 14-3-3 protein diverges considerably from the known classes of vertebrate 14-3-3 isoforms.

Three-dimensional structure of 14-3-3.a

The three-dimensional structure of the 14-3-3.a gene product was modeled using a protein structure algorithm (Peitsch, 1996) and experimentally determined structural coordinates of human and bovine 14-3-3ζ (Liu et al., 1998; Petosa et al., 1998; Rittinger et al., 1999) as templates. This was done to reveal whether the inside orientation and arrangement of the helices that form the amphipathic substrate binding groove are conserved in 14-3-3.a. Outside of the four conserved helices forming this important groove (H3, H5, H7 and H9) the linker regions connecting these helices show considerable variation from those of mammalian 14-3-3 proteins (Fig. 4). Nevertheless, the overall folding of the polypeptide chain and three-dimensional structure of *F. heteroclitus* 14-3-3.a is highly conserved compared to mammalian 14-3-3ζ (Fig. 5; Liu et al., 1995; Rittinger et al., 1999). Moreover, the three-dimensional arrangement of the four helices forming the amphipathic substrate-binding groove is very similar to those in 14-3-3ζ (Fig. 5; Petosa et al., 1998). These four helices form the inner face of 14-3-3.a and are in a perfect position to bind substrates in the same way as other 14-3-3 proteins. Based on these results it is nearly certain that *F. heteroclitus* 14-3-3.a interacts with the same substrates as 14-3-3ζ and other 14-3-3 proteins, i.e. preferentially with phosphoserine-containing polypeptides (Yaffe et al., 1997).

Osmotic regulation and tissue-specific expression of the 14-3-3.a gene

Northern blot analysis of *F. heteroclitus* 14-3-3.a mRNA expression in gill epithelium of fish acclimated to SW, FW, or 2× SW shows that this gene is osmoregulated at the level of mRNA abundance (Fig. 6A). Even though the abundance of 14-3-3.a mRNA in gill epithelium was unchanged at 24 h after transfer of fish from SW to 2× SW, it increased fourfold at 24 h after transfer of fish from SW to FW (Fig. 6A,C). This increase was highly statistically significant ($P<0.001$), and may result from either increased transcription or enhanced mRNA stability. Currently it is not known which mechanism underlies the osmotic regulation of 14-3-3.a mRNA abundance. Whatever the molecular basis for the increase of 14-3-3.a mRNA abundance after transfer of fish from SW to FW, it is not linearly correlated with external salinity (Fig. 6C). The transcriptional induction of 14-3-3.a is followed by induction at the protein level (Fig. 7). Western blot analysis revealed two 14-3-3 variants that are recognized by an antibody made against a synthetic peptide representing an epitope that is 100 % conserved in fish 14-3-3.a (Fig. 7A). Thus, the two bands may represent different post-translational variants of 14-3-3.a. Alternatively, they may represent the teleost homologs of

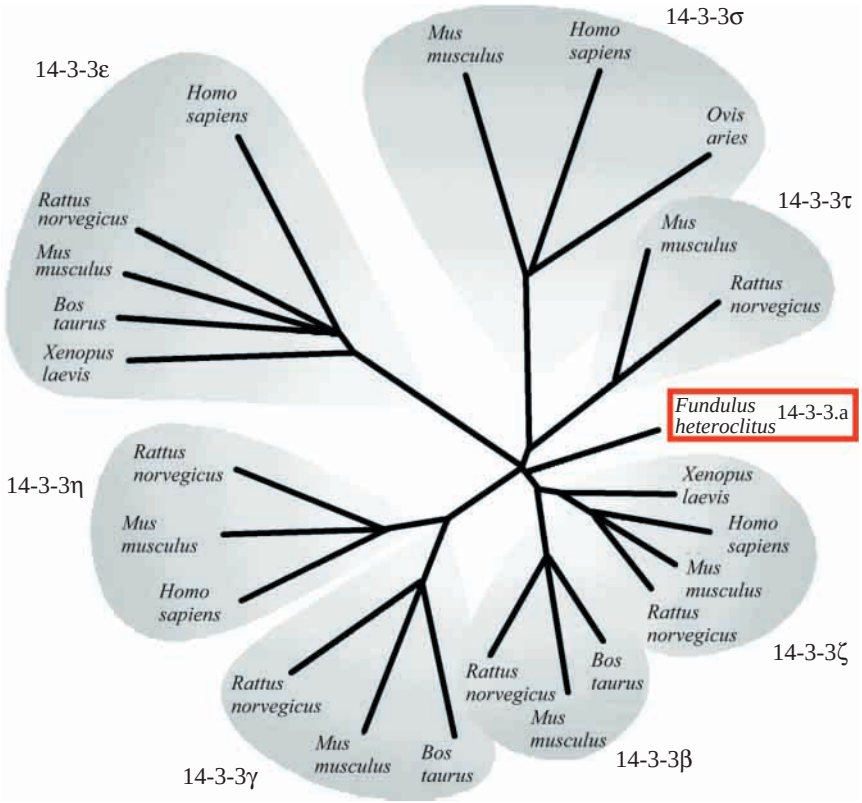

Fig. 3. Radial phylogenetic tree depicting the evolutionary relationship of *Fundulus heteroclitus* 14-3-3.a to the seven known mammalian and some *Xenopus laevis* 14-3-3 isoforms. 14-3-3 proteins from different vertebrates (labeled with species name) that are of the same isoform-type (either α, β, γ, ε, η, σ, or τ) cluster together (gray background). Such clusters are labeled with the isoform-type of the corresponding 14-3-3 proteins in each cluster. *F. heteroclitus* 14-3-3.a (boxed in red) may be evolutionarily distinct from the 14-3-3 isoforms that are known from higher vertebrates or be a homolog of 14-3-3ζ or 14-3-3β.

mammalian 14-3-3β and 14-3-3ζ. In this case, only one of the two bands would represent 14-3-3.a. Nevertheless, both 14-3-3 bands are significantly induced after transfer of fish from SW to FW, in accordance with the induction of 14-3-3.a at the level of mRNA abundance (Fig. 7B). Even though the mRNA levels of 14-3-3.a quadruple within 24 h of hyposmotic stress in the form of transfer of fish from a plasma-hyperosmotic (SW) to a plasma-hyposmotic (FW) environment, the baseline level in gill epithelium of SW fish is already much higher than in any other tissue examined (Fig. 6B,D). The baseline level of 14-3-3.a mRNA abundance in gill epithelium of SW fish is approx. threefold higher than in brain, where 14-3-3 proteins are thought to be generally most abundant (Fig. 6D). The abundance of 14-3-3.a mRNA in testes and liver is similar to that in brain while it is very low in ovary and almost not detectable in white muscle (Fig. 6B,D). The second highest levels of mRNA abundance are present in intestine, which, in addition to the gill, is another important osmoregulatory tissue for teleosts. The intestine is also the only other tissue where 14-3-3.a mRNA abundance significantly changes after salinity transfer of fish. However, in contrast to gill epithelium, transfer of fish from SW to FW had no effect while transfer of fish from SW to 2× SW leads to a significant (P<0.05) decrease of 14-3-3.a mRNA abundance in intestine (Fig. 6D). The high abundance and preferential osmotic regulation of 14-3-3.a in osmoregulatory tissues clearly indicates that this novel 14-3-3 gene plays an important osmoregulatory role for the euryhaline fish *F. heteroclitus*, in particular in the gill epithelium.

Discussion

14-3-3.a is a novel vertebrate 14-3-3 gene

14-3-3 proteins represent a novel type of adapter/scaffold protein that works as a molecular switch and modulates interactions between components involved in many signal transduction pathways to induce a rapid change from one type of metabolism to another in response to a change in environment (Baldin, 2000; Van der Hoeven et al., 2000; Aitken, 1995). A striking and well-documented feature of 14-3-3 proteins is their ability to bind a multitude of functionally diverse signaling proteins, including many kinases and phosphatases (Fu et al., 2000). Therefore, the cloning of *F. heteroclitus* 14-3-3.a and our discovery of its osmotic regulation should facilitate the identification of kinases, phosphatases and other proteins participating in osmosensory signal transduction, based on their association with 14-3-3.a. Such efforts could be crucial

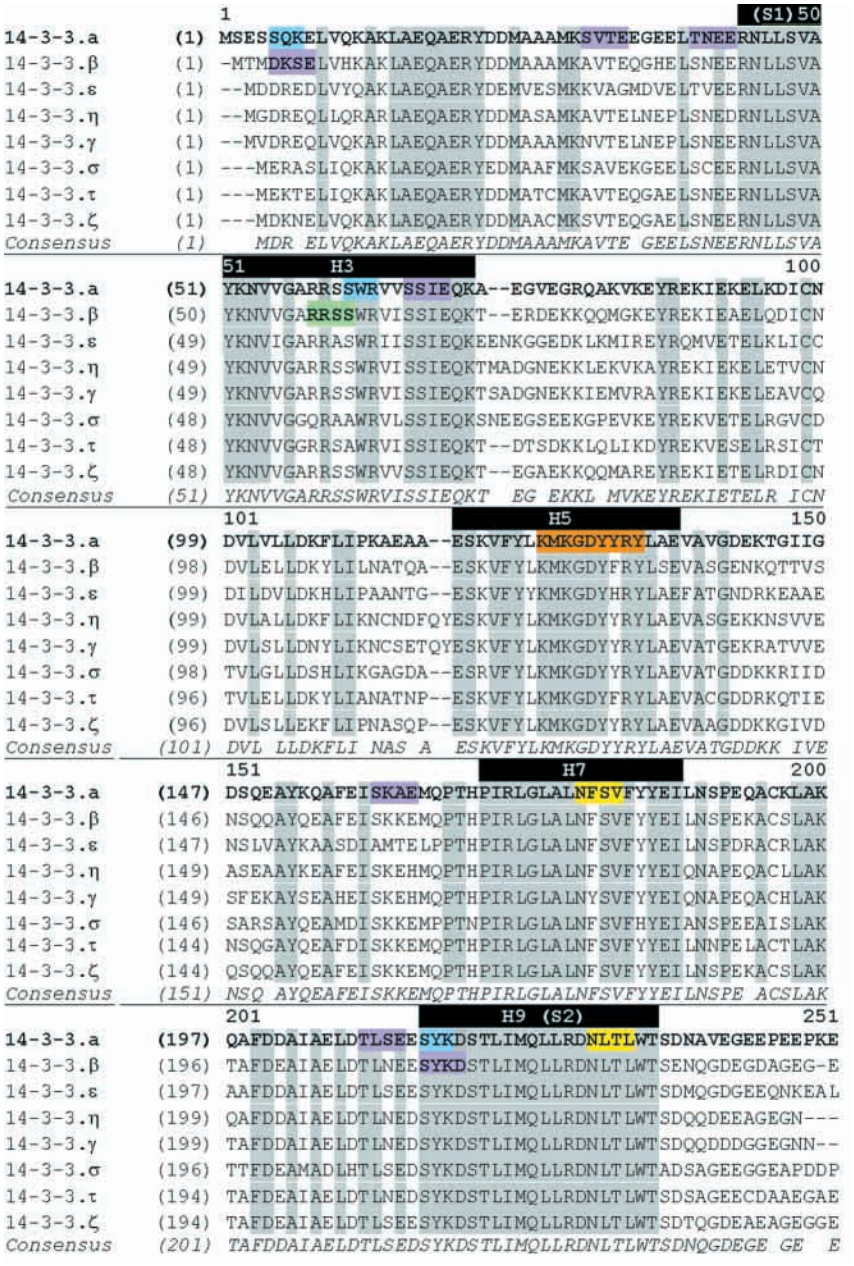

Fig. 4. Multiple sequence alignment of the amino acid sequences of *Fundulus heteroclitus* 14-3-3.a (in bold) and all seven isoforms of 14-3-3 from mammals (*Mus musculus*). Amino acid residues that are identical in all the 14-3-3 proteins are shown on gray background. The locations of helices 3, 5, 7 and 9 are indicated by black bars above the corresponding residues. These regions, which form the amphipathic substrate binding groove, are highly conserved in their amino acid sequence. Important domains are indicated by colored background: blue, protein kinase C phosphorylation site; purple, casein kinase II phosphorylation site; green, protein kinase A phosphorylation site; orange, tyrosine kinase phosphorylation site; yellow, Asn glycosylation site. Please note that the corresponding residues are colored in the 14-3-3β sequence when two sites utilize the same residues in the 14-3-3.a sequence.

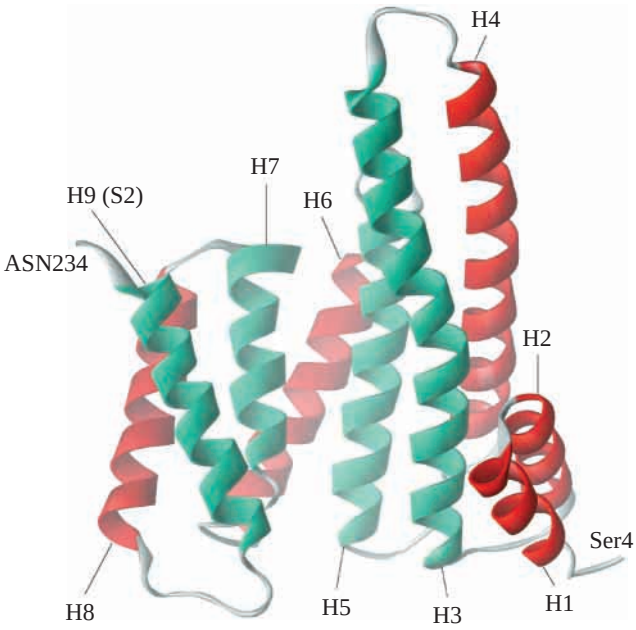

Fig. 5. Three-dimensional structure model of *Fundulus heteroclitus* 14-3-3.a. The protein structure is shown from Ser 4 to Asn 234 as a ribbon model with gray loop regions and colored helices. The first 3 amino acids of the amino terminus and the last 13 amino acids of the carboxy terminus are not shown because they are too different from the 14-3-3 proteins that have been used as templates during the modeling process. Helices are labeled (H1–H9). H9 corresponds to the 14-3-3 signature motif 2 (S2). Red helices form the outer coat of the protein while the four green helices (H3, H5, H7, H9) form the amphipathic substrate binding groove. The structural arrangement of the substrate binding groove is highly conserved in 14-3-3.a, suggesting that it binds the same set of phosphorylated proteins as other 14-3-3 proteins despite its overall distinct primary structure.

for understanding the molecular basis of salinity adaptation in teleost fish, and perhaps in salt-stressed cells of most organisms.

Evolutionary relationship of 14-3-3.a to other 14-3-3 proteins

In this study we have cloned and sequenced the first 14-3-3 gene from a euryhaline fish. The deduced amino acid sequence of this gene contains both of the 14-3-3 signature motifs: ^{44}RNL[LI]SVAYKN[VI] in helix 3 and ^{213}[SM]YK[DE]STLIMQLL[RH]DNLTLW[TA][SA] in helix 9 (Fig. 4; Wang and Shakes, 1996). Our phylogenetic analysis of the *F. heteroclitus* 14-3-3.a cDNA and deduced amino acid sequence indicates that it is related to 14-3-3 isoforms of mammals and amphibians, mostly to 14-3-3ζ (Fig. 3). Although 14-3-3 proteins are present in all eukaryotes and represent a large and highly evolutionarily conserved protein family, the number of genes contained in a genome differs greatly depending on species. For example, yeast, *Caenorhabditis elegans* and *Drosophila melanogaster* have only two 14-3-3 isoforms, while mammals have seven, and the plant *Arabidopsis thaliana* has ten isoforms of 14-3-3 (Wang and Shakes, 1996; Fu et al., 2000). Phylogenetic analysis indicates an early separation of plant from animal 14-3-3 isoforms (Wang and Shakes, 1996) and 14-3-3 gene duplication events may have occurred multiple times during evolution. Based on the phylogenetic analysis of fish 14-3-3.a it is possible that one 14-3-3 gene duplication event occurred during the transition from fish to amphibians, giving rise to paralogous 14-3-3β and 14-3-3ζ genes. However, it is not unusual that teleost genes are quite divergent from their mammalian homologs. Examples include catfish estrogen receptors (Xia et al., 2000; Hawkins et al., 2000) and the CFTR Cl^- channel that is important for salt secretion across teleost gills (Singer et al., 1998). Thus, it is also possible that fish 14-3-3.a represents a homolog of either mammalian 14-3-3ζ or 14-3-3β. Further studies are required to be able to decide whether *F. heteroclitus* 14-3-3.a represents the precursor gene giving rise to 14-3-3ζ and 14-3-3β or the fish homolog of only one of these 14-3-3 isoforms.

Structure of 14-3-3.a and putative substrates

The primary structure of *F. heteroclitus* 14-3-3.a cDNA is most similar to that of human 14-3-3ζ (Zupan et al., 1992) and frog 14-3-3ζ (Miura et al., 1997). All three sequences have a high degree of identity, not only in the CDS but also in the long 3′ UTR. The high degree of sequence identity in the 3′ UTR suggests that this region is important for post-transcriptional regulation of 14-3-3.a and 14-3-3ζ, but direct experimental evidence for this is currently lacking. The very high degree of structural conservation of the substrate-binding groove in fish 14-3-3.a provides strong evidence that this novel 14-3-3 protein binds the same substrates as mammalian 14-3-3 isoforms, i.e. proteins phosphorylated on Ser and, less commonly, Thr in a sequence-specific manner (Fu et al., 2000). 14-3-3 proteins form homo- or heterodimers and are generally most variable in the N and C termini (Finnie et al., 1999). This is consistent with the very low evolutionary conservation of the N- and C-terminal ends of *F. heteroclitus* 14-3-3.a, which contrasts with the high degree of sequence similarity in the four helices that form the amphipathic substrate-binding groove. Because 14-3-3 proteins are homo- or heterodimers with two substrate-binding sites (one for each monomer) they can promote protein–protein interactions, e.g. the interaction between the mitogen-activated protein (MAP) kinase activator Raf-1 and Bcr (Braselmann and McCormick, 1995). In contrast, a single ligand may be bound to 14-3-3 if it contains a dual recognition motif, perhaps to stabilize the interaction between 14-3-3 and ligand (Fu et al., 2000). In any case, the cloning of *F. heteroclitus* 14-3-3.a now makes it possible to identify its binding partners at different times during salinity acclimation of euryhaline fish and, therefore, obtain insight into osmosensory signal transduction of salt-tolerant gill epithelial cells *in vivo*.

Osmotic regulation of 14-3-3.a

"14-3-3 proteins are found in association with key control enzymes of primary metabolism, regulation of which could rapidly alter metabolic flux in response to signals such as

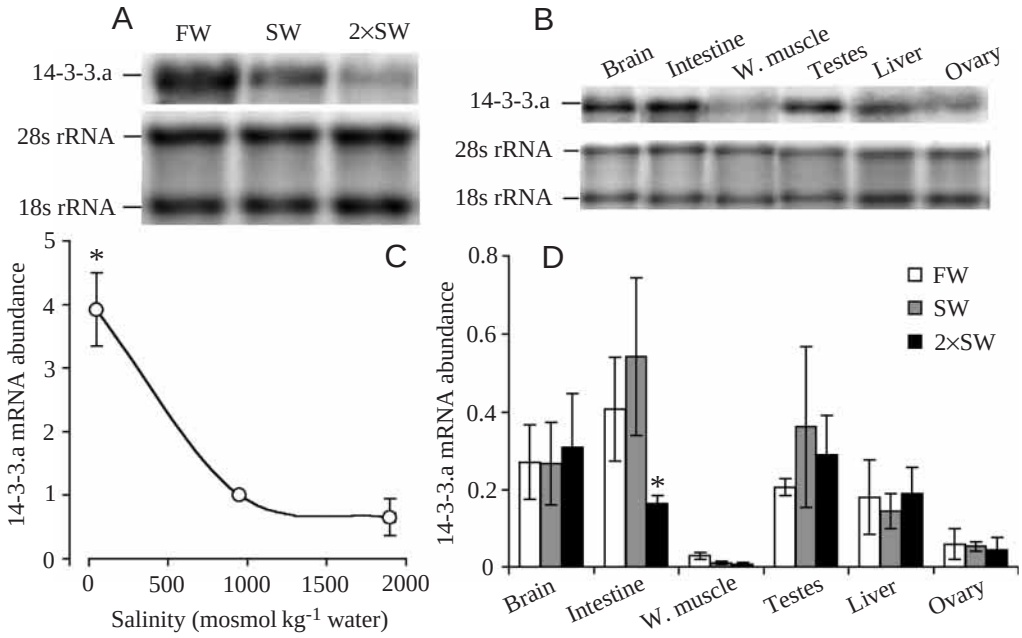

Fig. 6. Tissue-specific and osmotic regulation of 14-3-3.a gene transcription. 14-3-3.a mRNA abundance in various tissues of *Fundulus heteroclitus* was measured using northern blot analysis. (A) Northern blot of 14-3-3.a from gill epithelium RNA of *F. heteroclitus* acclimated to FW, SW and 2×SW for 24 h. Below the 14-3-3.a mRNA blot are the corresponding bands for the two rRNAs that were used for normalization. (B) Northern blot of 14-3-3.a with samples from different tissues of *F. heteroclitus* acclimated to SW. Below the 14-3-3.a mRNA blot are the corresponding bands for the two rRNAs that were used for normalization. (C) Quantification of 14-3-3.a mRNA expression in gill epithelium of *F. heteroclitus* acclimated to various salinities for 24 h. Values are normalized for 14-3-3.a mRNA abundance in SW fish (=1) that represent the pre-acclimation conditions. Values are means ± S.E.M., *N*=4; an asterisk indicates a significant difference from SW data. (D) Quantification of the effect of salinity acclimation on 14-3-3.a mRNA abundance in different tissues of *F. heteroclitus*. Data are normalized for 14-3-3.a mRNA abundance in SW fish (=1). Values are means ± S.E.M., *N*=4, except for testes and ovary where *N*=2. An asterisk indicates a significant difference from SW data.

water, osmotic, or salt stress." (Finnie et al., 1999). Thus, 14-3-3 proteins can be expected to play a significant role for cellular osmoregulation and it is reasonable to hypothesize that they are osmotically regulated. The results of this study clearly demonstrate that fish 14-3-3.a is osmoregulated. The osmotic regulation of 14-3-3.a, taken together with higher levels of expression in osmoregulatory tissues such as gill epithelium compared to brain, suggests that fish 14-3-3.a is important for the regulation of phosphoprotein cascades involved in osmosensory signal transduction. In addition to their abundance, 14-3-3 proteins are regulated *via* phosphorylation. Sphingosine-dependent protein kinase 1 (SDK1) mediates 14-3-3 phosphorylation in response to changes in lipid metabolism (Fu et al., 2000). Interestingly, lipid metabolism and fatty acid synthesis patterns are regulated by environmental salinity in gills of euryhaline eels (Hansen and Abraham, 1979; Hansen and Abraham, 1983). This includes sphingomyelin metabolism, which is important for SDK1 activity and linked to salt transport in gills of euryhaline bass and eels (El Babili et al., 1996). Specifically, a change in the ceramide moiety of sphingomyelin occurs when euryhaline fish (bass, eels) are transferred from SW (37 p.p.t.) to diluted SW (10 p.p.t.) and the resulting changes in levels of free sphingosine and ceramide have been proposed to play a significant role for tissue-remodeling and chloride cell differentiation in euryhaline teleosts subjected to salinity change (El Babili et al., 1996). The induction of 14-3-3.a in gill epithelium of fish acclimated to FW suggests that it is important for establishing the molecular and cellular basis of salt absorption across the gill epithelium.

Role of 14-3-3 for the regulation of ion transport

Fish surrounded by FW need to actively absorb salt from the environment to account for passive losses that occur as a result of diffusion along a concentration gradient. According to the prevalent model of branchial Na^+ absorption in FW fish, Na^+ enters gill epithelial cells *via* an apical Na^+ channel that is electrically coupled to a plasma membrane H^+-ATPase (Avella and Bornancin, 1989). This H^+-ATPase has been localized to the apical membranes of both chloride cells and pavement cells (Lin and Randall, 1993; Lin and Randall, 1994). It is possible that chloride cells and pavement cells are electrically coupled and that the Na^+ channel is only present in a single cell type (Goss et al., 1992). After apical entry into the cell, Na^+ is transported across the basolateral membrane into the blood by the Na^+/K^+-ATPase. This process is mainly energized by the H^+-ATPase, while the Na^+/K^+-ATPase mainly energizes NaCl absorption across the gills of fish in SW. Accordingly, Na^+/K^+-ATPase activity is downregulated and H^+-ATPase activity is upregulated in gill epithelium of fish transferred from SW to

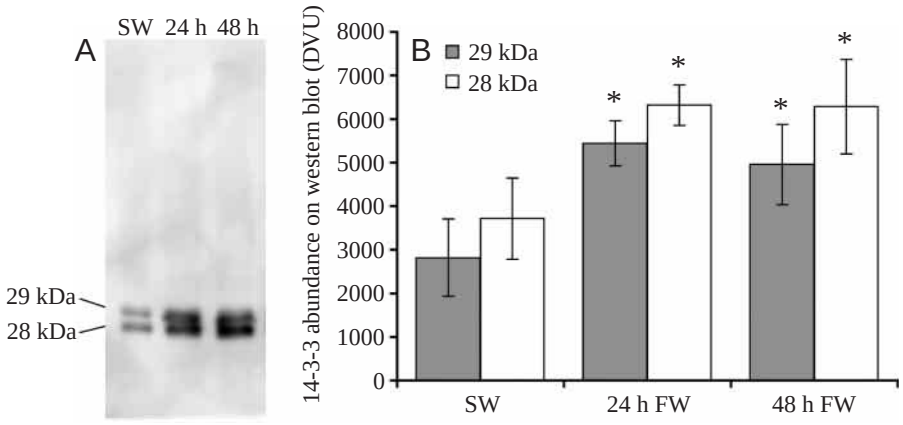

Fig. 7. Abundance of *F. heteroclitus* 14-3-3 protein in gill epithelium of fish acclimated to either SW or for 24 h or 48 h to FW. (A) Example of a western blot developed with an antibody against a synthetic peptide that is 100 % conserved in the N terminus of 14-3-3.a. This antibody recognizes two bands with apparent molecular mass of 28 kDa and 29 kDa. The abundance of both 14-3-3 bands increases after transfer of fish from SW to FW. (B) Quantification of 14-3-3 in gill epithelium of *F. heteroclitus* acclimated to SW and after transfer to FW. DVU, densitometric volume units; values are means ± S.E.M.; N=4. An asterisk indicates a significant difference to the respective 14-3-3 band in the SW sample (P<0.05).

FW (Kültz and Somero, 1995). Activation of H^+-ATPase, inhibition of Na^+/K^+-ATPase, and regulation of ion channels are all important functions of 14-3-3 proteins. In plant cells an increase in osmolality activates the H^+-ATPase fivefold and the 14-3-3 protein abundance in the plasma membrane two- to threefold, suggesting that 14-3-3 proteins are involved in the osmotic regulation of H^+-ATPase (Babakov et al., 2000). Indeed, virtually any 14-3-3 protein binds to phosphorylated Thr in the last 98 C-terminal amino acid residues (motif YTV) of plasma membrane H^+-ATPase and stimulates its activity in the presence of fusicoccin (Fuglsang et al., 1999; Baunsgaard et al., 1998). In addition, several 14-3-3 isoforms bind to and activate PKC (Isobe et al., 1992; Van der Hoeven et al., 2000), which in turn, inhibits Na^+/K^+-ATPase activity in gill epithelium of teleosts, e.g. Atlantic cod (Crombie et al., 1996). But 14-3-3 proteins not only affect the activity of ATPases, but are also very potent modulators of many ion channels. For instance, 14-3-3ε binds to calmodulin in a Ca^{2+}-dependent manner and inhibits the Ca^{2+}-activated Cl^-channel (CaCl) that is endogenously expressed in *Xenopus* oocytes (Chan et al., 2000). These authors suggest that activation of the CaCl channel by inhibition of 14-3-3ε may have therapeutic potential for circumventing the defective regulation of cAMP-activated Cl^- channels in cystic fibrosis. It is therefore possible that 14-3-3 proteins inhibit the activity of apical Cl^- channels in teleost chloride cells during acclimation of fish from SW to FW. These observations, combined with our results showing an increase of 14-3-3.a in gill epithelium of fish transferred from SW to FW, suggest that 14-3-3.a could be a major factor for the activation of H^+-ATPase, downregulation of Na^+/K^+-ATPase, and inhibition of the Cl^- channel in gill epithelial cells of euryhaline fish transferred from SW to FW and, therefore, represent a molecular master regulator for osmotic adaptation and the configuration of flexible ion transport mechanisms in the same tissue.

14-3-3 function for tissue remodeling and cell proliferation

The gill epithelium covering primary and secondary lamellae is extensively remodeled in response to transfer of euryhaline fish from hyposmotic to hyperosmotic environments or *vice versa* (Laurent and Dunel, 1980; Kültz et al., 1995). Such remodeling concerns both cellular differentiation and the properties of extracellular structures, for instance tight junctions (Hwang, 1987). As a consequence of increased proliferation and increased cell death, the turnover of chloride cells and pavement cells is higher in fish acclimated to SW than in fish kept in FW (Chretien and Pisam, 1986; Wendelaar Bonga, 1997; Uchida and Kaneko, 1996). Fish 14-3-3.a could be crucial for the salinity-dependent modulation of chloride cell and/ or pavement cell differentiation as well as for the observed cell cycle changes. 14-3-3 proteins interact with and regulate many mitogenic oncogene products, cell cycle regulators, apoptosis regulators, cell cycle checkpoint factors and transcription factors that control cellular differentiation programs (reviewed in Datta et al., 1999; Fu et al., 2000; Schultz et al., 1998). 14-3-3 proteins also regulate the actin cytoskeleton, vesicle-trafficking, and Ca^{2+}-dependent exocytosis (Chamberlain et al., 1995; Roth et al., 1999). This could be important for the shuttling of pumps and transporters to different membrane domains as well as for modulating the extent of basolateral membrane infoldings. Future investigations will reveal exactly which binding partners are modulated by fish 14-3-3.a and whether 14-3-3.a binding leads to activation, inhibition or translocation of these proteins during salinity acclimation of euryhaline fish.

In summary, we have cloned and analyzed the novel 14-3-3.a gene from the euryhaline teleost *Fundulus heteroclitus*. Because of its high abundance, osmotic regulation, and structural conservation compared to other 14-3-3 proteins, 14-3-3.a appears to be a molecular master regulator for osmosensory signal transduction in gill epithelial cells of euryhaline teleosts. Further investigation of its regulation and identification of phosphorylated binding partners during osmotic adaptation of teleost fish should greatly advance our understanding of the molecular basis for osmolality-dependent cell adaptation, not only in gill epithelium of euryhaline fish but in epithelia exposed to salinity changes in general.

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