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Separate Na,K-ATPase genes are required for otolith formation and semicircular canal development in zebrafish

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Abstract

We have investigated the role of Na,K-ATPase genes in zebrafish ear development. Six Na,K-ATPase genes are differentially expressed in the developing zebrafish inner ear. Antisense morpholino knockdown of Na,K-ATPase α1a.1 expression blocked formation of otoliths. This effect was phenocopied by treatment of embryos with ouabain, an inhibitor of Na,K-ATPase activity. The otolith defect produced by morpholinos was rescued by microinjection of zebrafish α1a.1 or rat α1 mRNA, while the ouabain-induced defect was rescued by expression of ouabain-resistant zebrafish α1a.1 or rat α1 mRNA. Knockdown of a second zebrafish α subunit, α1a.2, disrupted development of the semicircular canals. Knockdown of Na,K-ATPase β2b expression also caused an otolith defect, suggesting that the β2b subunit partners with the α1a.1 subunit to form a Na,K-ATPase required for otolith formation. These results reveal novel roles for Na,K-ATPase genes in vestibular system development and indicate that different isoforms play distinct functional roles in formation of inner ear structures. Our results highlight zebrafish gene knockdown-mRNA rescue as an approach that can be used to dissect the functional properties of zebrafish and mammalian Na,K-ATPase genes.

Keywords: Zebrafish; Na,K-ATPase; Sodium pump; Morpholino; Inner ear; Otolith; Semicircular canal; mRNA rescue

Introduction

Na,K-ATPase, also known as the sodium pump, is a key enzyme essential for maintaining cellular homeostasis. By transporting sodium and potassium ions, the sodium pump establishes and maintains electrochemical gradients that underlie electrical excitability of nerve and muscle and the transport of numerous solutes and water across epithelia (Thomas, 1972). The active enzyme is a major consumer of ATP, and is also the cellular receptor for cardiac glycoside drugs that have historically been used in the treatment of congestive heart failure and cardiac arrhythmias.

The sodium pump is composed of two functionally required subunits, α and β, which are present in equimolar amounts. A putative third subunit, termed γ, has been identified, although this subunit does not appear to be essential for Na,K-ATPase activity (Scheiner-Bobis and Farley, 1994). Multiple isoforms of each of the α and β subunits have been described in a variety of species. In mammals, four isoforms of the α subunit (Herrera et al., 1987; Shamraj and Lingrel, 1994; Shull et al., 1986) and three separate β subunit isoforms (Malik et al., 1996; Martin-Vasallo et al., 1989; Mercer et al., 1986) have been identified. Each α and β subunit is encoded by a distinct gene, and each gene is expressed in a unique tissue and developmentally regulated fashion. Most strikingly, in vitro experiments show

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that each α subunit is capable of interaction with any of the β subunits to form a functional enzyme (Crambert et al., 2000; Lemas et al., 1994; Schmalzing et al., 1997).

The potential for promiscuity in α/β subunit interactions raises the possibility for 12 different Na,K-ATPase isoenzymes in mammals. The situation in zebrafish is even more complex, with 9 α subunit and 6 β subunit genes having been identified (Blasiole et al., 2002; Rajarao et al., 2001, 2002). Thus, zebrafish have the potential for 54 possible α/β subunit combinations. A critical gap in our understanding of Na,K-ATPase diversity is whether the proteins encoded by each gene have unique or redundant physiological functions. One issue is that the different α/β subunit combinations produced in vitro all seem to have very similar biochemical properties (Crambert et al., 2000; Jewell and Lingrel, 1991). Attempts to address the question of Na,K-ATPase isoform diversity in knockout mice have been only partially successful, as homozygous null mutations at the α1 or α2 loci produce embryonic lethality (James et al., 1999), and homozyzous β2 mutant mice die shortly after birth (Magyar et al., 1994).

Several lines of evidence suggest that the question of Na,K-ATPase isoform diversity may be addressed in zebrafish. Experiments carried out by Shu et al. (2003) have shown that the zebrafish cardiac mutant *heart and mind* is caused by a defect in the Na,K-ATPase α1a.1 gene. The *heart and mind* mutant phenotype could be rescued by α1a.1 mRNA but not the zebrafish α2 subunit mRNA, indicating that these two Na,K-ATPase α subunits play distinct roles in zebrafish cardiac development and function.

In this report, we have investigated the role of several Na,K-ATPase genes in zebrafish inner ear development utilizing antisense morpholino gene knockdown. Knockdown of α1a.1, an α subunit gene previously shown to be required for normal myocardial (Shu et al., 2003) and brain ventricle (Lowery and Sive, 2005) development in zebrafish, resulted in otolith agenesis. Otoliths (or otoconia in mammals) are calcium carbonate deposits within the inner ear that facilitate the transmission of vibration and acceleration forces to macular hair cells, which is required for hearing and balance. Knockdown of a closely related α subunit, α1a.2, caused morphological defects in semicircular canals, which are inner ear structures responsible for sensing angular acceleration (Chang et al., 2004b).

The studies presented here indicate that different Na,K-ATPase genes are required for development of distinct components of the zebrafish inner ear. Our results highlight the utility of the zebrafish system for dissecting functional differences amongst zebrafish Na,K-ATPase isoforms. Rescue experiments of the type we describe show that zebrafish can be useful for exploring the functional significance of mammalian Na,K-ATPase gene diversity as well.

Materials and methods

Antisense morpholinos and phenotypic rescue

The antisense morpholinos (MOs; Gene Tools LLC; Philomath, OR) targeted against individual Na,K-ATPase α and β subunit genes are presented in Table 1. Two independent MOs were targeted against each gene. The MOs were resuspended in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) and injected into the yolk of single-cell zebrafish embryos. MOs were 3′ labeled with either FITC or Lissamine to monitor for uniform oligonucleotide distribution in injected embryos.

The ability of a MO to specifically block translation of its cognate mRNA was analyzed using an in vitro translation assay. Full-length mRNAs were transcribed from each Na,K-ATPase gene (Table 1) using the mMESSAGE mMACHINE transcription kit (Ambion; Austin, TX). Protein synthesis was initiated with the addition of 0.5 μg of mRNA to a rabbit reticulocyte lysate translation mix (Ambion). Proteins were labeled by the addition of [^{35}S]-methionine to the translation reaction. The translation of each mRNA was tested in the presence or absence of MOs (4 μM). The entire in vitro reaction mixture (20 μl) was loaded onto a 10% SDS-polyacrylamide gel and fractionated by electrophoresis. The gels were dried, exposed to X-ray film, and the relative intensity of the bands quantified by densitometry.

For phenotypic rescue, full-length open reading frames (ORFs) corresponding to zebrafish α1a.1, α1a.2, and rat α1 Na,K-ATPase genes were synthesized by PCR from cDNA constructs (Rajarao et al., 2001). Rescue mRNA constructs were designed with a minimal Kozak consensus sequence adjacent to the initiating ATG so as not to match the native 5′-UTR MO target sequence. Each PCR product was verified by DNA sequence analysis, subcloned into the zebrafish expression vector pT3TS (Hyatt and Ekker, 1999), and used to synthesize full-length capped mRNA. Rescue mRNAs were injected into one-cell stage embryos either alone or in the presence of an antisense MO. For each rescue experiment, the amount of mRNA injected was titrated for the maximal dose that could be injected without causing toxicity to embryos.

Ouabain treatment

Zebrafish embryos were treated with ouabain essentially as described (Shu et al., 2003). Briefly, wild-type zebrafish embryos were raised in charcoal-filtered

Table 1
Na,K-ATPase antisense morpholinos

Morpholino	Targeted gene[a]	Target[b]	Morpholino sequence
α1a.1 MO-1	*atp1a1a.1* (α1a.1)	−7 to +18	5′-gccttctcctcgtcccattttgctg-3′
α1a.1 MO-2	*atp1a1a.1* (α1a.1)	−32 to −8	5′-cttttgattaaaatcgaccaactgg-3′
α1a.2 MO-1	*atp1a1a.2* (α1a.2)	−20 to +5	5′-tgccttgttgacttgtttggagaca-3′
α1a.2 MO-2	*atp1a1a.2* (α1a.2)	−52 to −28	5′-cccatttttccaagtttatttcaacc-3′
β1a MO-1	*atp1b1a* (β1a)	−1 to +24	5′-gtcaccatctttatttgcgggcatt-3′
β1a MO-2	*atp1b1a* (β1a)	−28 to −4	5′-cggtatttagttccctttttggtgg-3′
β2b MO-1	*atp1b2b* (β2b)	−25 to −1	5′-tttcctggactaaatgtcgctcac-3′
β2b MO-2	*atp1b2b* (β2b)	−78 to −54	5′-ctttgagagtaataaagagctattc-3′
Standard Control			5′-cctcttacctcagttacaatttata-3′

[a] The GenBank accession numbers for the genes are as follows: α1a.1, AF286372; α1a.2, AF286374; β1a, AF286375; β2b, AF373976.
[b] Target sequence of mRNA with $^{+}$1 corresponding to the adenosine of the initiating methionine.