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Tissue-specific variation in 5′-terminal exons of mouse Anoctamin 1 transcript induces N-terminal variation of its protein via alternative translational start sites

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ABSTRACT

Anoctamin 1 (encoded by the *Ano1* gene) is a Ca^{2+}-activated Cl^- channel critical to many physiological functions. It has been speculated that *Ano1* expression is regulated in a tissue-dependent manner via alternative promoters. However, variation in the 5′-end sequence of mouse *Ano1* (m*Ano1*) and its tissue-dependent regulation are poorly understood. We identified a novel 5′-terminal exon (designated exon 1a) of m*Ano1* instead of the known 5′-terminal exon (exon 0) using 5′-rapid amplification of cDNA ends (RACE) analysis. Unexpectedly, the novel 5′-end variant m$Ano1_{Ex1a}$ was abundantly expressed in many tissues including the salivary and mammary glands, rectum, lung, trachea and prostate. In contrast, the known variant m$Ano1_{Ex0}$ predominated only in male reproductive tissues such as the epididymis and testis. In a heterologous expression system, m$Ano1_{Ex0}$ encoded a longer protein than m$Ano1_{Ex1a}$, and this long isoform was abolished by a mutation in the exon 0 start codon. Moreover, the m$Ano1_{Ex0}$-specific N-terminal sequence was immunohistochemically detected in epididymis but not in salivary gland. Our data suggest that m*Ano1* expression is regulated via alternative promoters, and its transcriptional variation results in variation of the N-terminal sequence of the Ano1 protein due to the alternative translation initiation sites. These tissue-specific variations might contribute to the regulation of m*Ano1* expression and activity according to the physiological function of each tissue.

1. Introduction

Anoctamin 1 (ANO1, synonymous with TMEM16A) is encoded by *Ano1* in mice or *ANO1* in humans and functions as a Ca^{2+}-activated Cl^- channel (CaCC) [1]. Growing evidence has indicated that ANO1 is critical to a variety of physiological functions including gastrointestinal motility, sensation, and ionic fluid secretion from exocrine glands [2].

The alternative splicing of exons (e.g., exon 6b, exon 13, and exon 15) produces numerous variants of human *ANO1* (h*ANO1*) transcripts [2]. The inclusion and skipping of these exons modulate channel activities such as Ca^{2+} sensitivity and time-dependent activation kinetics [3]. Furthermore, the variation of the 5′-terminal exon of h*ANO1* is probably induced by alternative promoters (Fig. S1). Sondo et al. [4] revealed an h*ANO1* variant predominantly expressed in testis possessing an alternative 5′-terminal exon to the common human 5′-terminal exon (exon 1). Mazzone et al. [5] demonstrated the increased expression of a variant lacking two exons (exons 1 and 2) and possessing a longer exon 3 in gastric biopsies from patients with diabetic gastroparesis. They also identified another h*ANO1* variant with an additional exon upstream of exon 1 in healthy gastric smooth muscle [6]. Some of these 5′-end variants of h*ANO1* caused variation in the N-terminal sequence of the translated protein via alternative translational initiation sites (TISs), possibly altering the molecular function [4–7]. These findings suggest that variation in the 5′-terminal exon of *Ano1* contributes to tissue-specific physiological functions and pathophysiological malfunction by modulating the transcriptional activity and channel function.

Although mice are useful animal models for investigating the role of ANO1, an understanding of the variations in 5′-end of mouse

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Ano1 (m*Ano1*) mRNA and the N-terminal sequence of mANO1 protein remains unclear and confusing. The 5′-end sequences of the first exon of two m*Ano1* reference sequences (NM_178642.5 and NM_001242349.1) are identical to each other and are less homologous to any reported human first exons. The start codon in the second exon and its downstream sequence of the m*Ano1* reference sequence is homologous to the start codon in the exon 1 and its downstream sequence of the h*ANO1*. Thus, in this paper, we have designated the first and second exons of the m*Ano1* reference sequences as “exon 0” and “exon 1,” respectively (Fig. S1). A former version of the m*Ano1* reference sequence (NM_178642.4) had a shorter exon 0 that lacked a start codon. Because of its homology with human *ANO1*, the start codon in m*Ano1* exon 1 was believed to be the major TIS for the mANO1 protein. Thus, in most studies addressing mANO1 function using a heterologous expression system, an m*Ano1* construct lacking a start codon in exon 0 was used [8–11]. However, the reference sequence of m*Ano1* was recently updated to elongate exon 0 toward its 5′-end by 114 nucleotides that contain a start codon. If the start codon in exon 0 were functional, m*Ano1* with exon 0 (m$Ano1_{Ex0}$) would generate an mANO1 protein with additional 57 amino acids at the N-terminal (mANO1$_{Met1}$) compared with the mANO1 protein translated from the start codon in exon 1 (mANO1$_{Met58}$). The predominant variant of m*Ano1* mRNA and the isoforms of mANO1 protein in tissues as well as their regulation remain obscure.

The present study aimed to resolve these perplexities by reevaluating the 5′-terminal exon of m*Ano1* with 5′-rapid amplification of cDNA ends (RACE) analysis of cDNA obtained from mouse mammary glands where we had previously detected ANO1 expression and an ANO1-like current [12]. We also measured the tissue-dependent expression of the 5′-end variants using real-time PCR. Then, possible variations in the N-terminal sequence of translated m*Ano1* were examined using a heterologous expression system, Western blotting, and immunohistochemistry.

2. Material and methods

2.1. Animals

C57BL/6J Jms Slc mice were obtained from Nihon SLC (Shizuoka, Japan). The experimental procedures and animal care were performed as per the Regulations on Management and Operation of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine (OUAVM) and were approved by the OUAVM Animal Care and Use Committee.

2.2. 5′-RACE analysis

The lactating mice were killed by cervical dislocation, and their mammary glands were collected in RNAlater® solution (Life Technologies, Carlsbad, CA, USA). The total RNA was extracted from the tissues with TRIzol® reagent (Life Technologies) and was purified with an RNeasy (QIAGEN, Hilden, Germany) or a NucleoSpin RNA Clean-up kit (Macherey-Nagel, Düren, Germany). 5′-RACE analyses were performed using a 5′-Full RACE Core Set (Takara, Shiga, Japan) according to the manufacturer's instructions. Briefly, the total RNA was reverse-transcribed with a 5′-phosphorylated m*Ano1*-specific primer (5′-(P)tgtagtcccctcct-3′) and avian myeloblastosis virus reverse transcriptase. The single-stranded cDNA that contained an unknown sequence at the 5′-end region of m*Ano1* was ligated with T4 RNA ligase to obtain circular or concatemeric cDNA. Then, the sequence of interest was amplified by nested PCR with LA Taq® DNA polymerase (Takara) and specific primers (Table S1). The DNA fragment was subcloned by bacterial transformation and colonization with a pGEM®-T Easy Vector System (Promega, Madison, WI) and was sequenced (Sigma-Aldrich Japan, Tokyo, Japan). The web-based program ATGpr (http://atgpr.dbcls.jp/) was used to predict the possible TISs in the m*Ano1* variants.

2.3. Reverse-transcription (RT)-PCR

Tissues were collected from male (9–10 weeks old), virgin female (10 weeks old), and lactating female mice (16–17 weeks old). The total RNA was isolated and purified from the tissues and was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) [12]. The RT product was employed for PCR in a reaction buffer containing Go Taq® polymerase (Promega) for short fragments (<500 bp) or PrimeSTAR® HS DNA polymerase (Takara) for long fragments (>3 kbp). The primers used are listed in Table S1.

Real-time PCR was performed in a LightCycler® 480 Instrument II (Roche, Mannheim, Germany) using SYBR Green as the double-strand DNA-specific dye (KAPA SYBR® FAST qPCR Kit; Nippon Genetics, Tokyo, Japan) according to the manufacturer's protocol. Fluorescence was measured in every amplification cycle (10 s at 95 °C for denaturing, 20 s at 60 °C for annealing and extension, and 1 s at 72 °C for data acquisition), and the values for the threshold cycle (Ct) were determined for the standards (pGEM®-T Easy Vectors containing target fragments) and samples. The amount of the target sequence in a sample was determined from the standard curve of Ct values. The expression levels of mAno1 variants were determined as relative values to *Actb*. The experiments were performed in duplicate, and the primers used are listed in Table S1.

2.4. Heterologous expression of mAno1 variants

The full-length coding sequence (CDS) of m$Ano1_{Ex0}$ was amplified from cDNA of mouse testis using PrimeSTAR® HS DNA polymerase and specific primers (Table S1). Briefly, the amplified fragment was inserted into a pGEM®-T Easy Vector, and the cloned m$Ano1_{Ex0}$ sequence was then inserted into a pCI-neo Mammalian Expression Vector (Promega) to obtain an m$Ano1_{Ex0}$-expression vector. Depending on the predominant m*Ano1* spliced variant in the mouse testis samples, the m$Ano1_{Ex0}$-expression vector contained exon 13 but not exon 6b and exon 15 (m*Ano1*[*Ex0*, *ΔEx6b*, *Ex13*, and *ΔEx15*]). To examine the role of the start codon in exon 0, the *ATG* codon of the pGEM-mAno1$_{Ex0}$ vector was substituted by *ATC* using a PrimeSTAR® Mutagenesis Basal kit (Takara) and mutagenic primers (sense, 5′-cacatcatccaggacgcgcaggacagc-3′; antisense, 5′-gtcctggatgatgtgttcgctccggac-3′). Then, the mutated m$Ano1_{Ex0}$ was inserted into a pCI-neo mammalian expression vector to create an m$Ano1_{Ex0}$ *(c.3G > C)*-expression vector. The full CDS of m$Ano1_{Ex1a}$ was cloned from cDNA obtained from submandibular gland tissue. Because this variant contained exon 6b, the exon was removed using restriction enzyme *Stu*I (Takara) and DNA ligase (Ligation High; Toyobo, Osaka, Japan). Finally, m*Ano1* (*Ex1a*, *ΔEx6b*, *Ex13*, and *ΔEx15*) was inserted into the expression vector.

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal calf serum. The m$Ano1_{Ex0}$-, m$Ano1_{Ex1a}$-, and m$Ano1_{Ex0}$ *(c.3G > C)*-expression vectors (2.5 μg) were transfected into subconfluent HEK293 cell cultures in a six-well plate with Lipofectamine® 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and cultured for 2 days. Then, the cells were washed with ice-cold phosphate-buffered saline and harvested with a lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM $NaVO_3$, 5 mM EDTA, 1% Nonidet P-40, and a protease inhibitor cocktail [cOmplete™; Roche]). After incubating the lysed cells on

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