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Muscle regulatory factors regulate T1R3 taste receptor expression

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ABSTRACT

T1R3 is a T1R class of G protein-coupled receptors, composing subunit of the umami taste receptor when complexed with T1R1. T1R3 was originally discovered in gustatory tissue but is now known to be expressed in a wide variety of tissues and cell types such the intestine, pancreatic β-cells, skeletal muscle, and heart. In addition to taste recognition, the T1R1/T1R3 complex functions as an amino acid sensor and has been proposed to be a control mechanism for the secretion of hormones, such as cholecystokinin, insulin, and duodenal HCO_3^- and activates the mammalian rapamycin complex 1 (MTORC1) to inhibit autophagy. *T1R3* knockout mice have increased rate of autophagy in the heart, skeletal muscle and liver. Thus, T1R3 has multiple physiological functions and is widely expressed *in vivo*. However, the exact mechanisms regulating T1R3 expression are largely unknown.

Here, we used comparative genomics and functional analyses to characterize the genomic region upstream of the annotated transcriptional start of human *T1R3*. This revealed that the *T1R3* promoter in human and mouse resides in an evolutionary conserved region (ECR). We also identified a repressive element located upstream of the human *T1R3* promoter that has relatively high degree of conservation with rhesus macaque. Additionally, the muscle regulatory factors MyoD and Myogenin regulate *T1R3* expression and *T1R3* expression increases with skeletal muscle differentiation of murine myoblast C2C12 cells.

Taken together, our study raises the possibility that MyoD and Myogenin might control skeletal muscle metabolism and homeostasis through the regulation of *T1R3* promoter activity.

1. Introduction

The sense of taste acts as the guardian and guide for food intake and is essential for body maintenance [1]. Taste reception systems have changed during the evolutionary process with noted variability in taste bud location, sensitivity to certain substances, and the expression patterns of taste receptors and their binding systems [2–6]. T1R3 is a bifunctional sensor of the T1R class of G protein-coupled receptors, composing one subunit of either the umami or sweet taste receptor when complexed with T1R1 or T1R2, respectively [7–10]. T1R3 was originally discovered in gustatory tissue [9] but is now known to be expressed in a wide variety of tissues and cell types such the intestine, pancreatic β-cells, skeletal muscle, and heart [11–14]. In addition to taste recognition, the T1R1/T1R3 complex functions as an amino acid sensor and has been proposed to be a control mechanism for the secretion of hormones, such as cholecystokinin, insulin, and duodenal HCO_3^- [15] and activates the mammalian rapamycin complex 1 (MTORC1) to inhibit autophagy. *T1R3* knockout mice have increased rate of autophagy in the heart, skeletal muscle and liver [14,16].

Although T1R3 has multiple physiological functions and is widely expressed *in vivo*, the exact mechanisms regulating T1R3 expression are largely unknown. Here, we utilize comparative and functional genomics to characterize the genomic region upstream

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of *T1R3* in mammals. We identified two distinct evolutionary conserved regions (ECRs), one of which functions as the *T1R3* promoter and the other as a repressor of human *T1R3* expression. Furthermore, we demonstrate that the muscle regulatory factors MyoD and Myogenin regulate *T1R3* expression and that the expression level of *T1R3* increases with myogenic differentiation of murine myoblasts.

2. Materials and methods

2.1. In silico experiments

DNA sequences were aligned using BLASTN [17] Version 2.2.26 + or ECR Brower [18] through the respective online servers or locally using MUSCLE in MEGA5 software [19]. The consensus sequence upstream of *T1R3* was constructed using the Los Alamos National Laboratory's Simple Consensus Maker (http://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html) using "Output aligned" parameter. For identification of transcription factor binding sites, DNA sequences were first aligned using zPicture [20] then transferred to rVista 2.0 [21].

2.2. Plasmids

cDNA encoding murine *Myogenin* (accession number NM_031189.2) was obtained by a standard RT-PCR technique using PrimeSTAR HS DNA polymerase (TaKaRa, Ohtsu, Japan) from cDNA library of murine myoblast cell line, C2C12 cells [22] and cloned into FLAG-tagged pcDEF3 expression vector [23]. Sequences corresponding to specific regions upstream of human *T1R3* (−2.447 to 0, −1.330 to 0, −0.844 to 0, and −0.439 to 0) were amplified from genomic DNA of HEK293T cells and cloned into the promoter-less firefly luciferase reporter vector pGL4.14 (Promega, Madison, WI). For some experiments, sequences corresponding to the specific regions upstream of human *T1R3* (−2.447 to −1.330, −1.330 to −0.844, −0.844 to −0.439, and −0.439 to 0) were subcloned from the Human *T1R3* (−2.447 to 0) plasmid upstream of the minimal thymidine kinase promoter in pGL4.26 (Promega). Elsewhere, sequences corresponding to specific regions upstream of human *T1R3* (−2.447 to −1.330, −2.447 to −2.170, −2.170 to −1.784, −1.784 to −1.330, and −1.330 to −0.844) were subcloned upstream of the human *T1R3* promoter (−0.439 to 0) in pGL4.14 (Promega). Sequences corresponding to specific regions upstream of mouse *T1R3* (−1.910 to 0, −1.530 to 0, −0.740 to 0, and −0.480 to 0) were amplified from genomic DNA of mouse tissue and cloned into pGL4.14. All of the final constructs were confirmed by sequencing. A plasmid encoding FLAG-tagged murine MyoD was kindly provided by Dr. Takenobu Katagiri (Saitama Medial University).

2.3. Cell culture, transfection, and luciferase assay

HEK293T cells, C2C12 cells, and C3H10T1/2 cells were cultured and maintained as described previously [24,25]. Cells were transfected with plasmids using Lipofectamine 3000 (Invitrogen) according to manufacturer's instruction. Luciferase assays were performed using each human or murine T1R3 luciferase plasmid and phRL-SV40 (Promega) with the Dual-Glo Luciferase Assay System (Promega) as previously described [26].

2.4. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed with a ChIP assay kit (Active Motif Carlsbad, CA) according to the manufacturer's instructions using anti-FLAG antibody (WAKO, Osaka, Japan) and normal mouse IgG (MBL, Aichi, Japan). The purified DNA was analyzed by PCR using primers targeting the *T1R3* promoter region and (−0.480 to 0) (5′-aaggttagaccagctagcc-3′ and 5′-tggtaggtagaagtctccag-3′).

2.5. Immunoblotting

C2C12 cells were lysed in TNT buffer (20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol) containing protease inhibitors (Roche) [27]. The following antibodies were used for immunoblotting: anti-T1R3 polyclonal antibody (N-20; sc-22458; Santa Cruz), anti-Myogenin polyclonal antibody (F5D, Santa Cruz), and anti-β-actin mouse monoclonal antibody (Sigma). Blots were washed in buffer containing 10 mM Tris–HCl, 50 mM NaCl, and 0.25% Tween 20) and incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling). The immunoreactive proteins were visualized using ECL (Amersham Biosciences, Piscataway, NJ).

2.6. Isolation of total RNA, reverse transcription, and real-time PCR analysis

Total RNA was isolated from C3H10T1/2 cells or C2C12 cells by using Trizol (Invitrogen) and then reverse-transcribed into cDNA using Superscript III (Invitrogen). The cDNA was amplified by PCR using specific primers for murine *MyoD* (5′-agcactacagtggcgactca-3′ and 5′-ggccgctgtaatccatcat-3′), murine *Myogenin* (5′-ccttgctcagctccctca-3′ and 5′-tgggagttgcattcactgg-3′), murine *Muscle creatine kinase (MCK)* (5′-cagcacagacagacactcagg-3′ and 5′-gaacttgttgtgggtgttgc-3′), murine *T1R3* (5′-aacagcatcccgtgcaac-3′ and 5′-ccacagccatcttcatagcc-3′), and murine *β-actin* (5′-aaggc-caaccgtgaaaagat-3′ and 5′-gtggtacgaccagaggcatac-3′). SYBR green-based quantitative real-time PCR was performed in 96-well plate using THUNDERBIRD qPCR mix (TOYOBO, Osaka, Japan) with a 7300 Real-time PCR system (Applied Biosystems) [28]. Values were normalized to *β-actin* using the $2^{(-\Delta\Delta Ct)}$ method [29].

2.7. Statistical analysis

Comparisons were made using an unpaired Student's t-test; the results are shown as means ± SD Statistical significance is indicated as #$p < 0.05$ and *$p < 0.01$.

3. Results

3.1. Conservation analysis of the T1R3 upstream region

We first used ECR Browser [20] to identify regions of nucleotide conservation between *Homo sapiens* (human) and *Macaca mulatta* (rhesus macaque), *Mus musculus* (mouse), *Rattus novegicus* (rat), *Canis lupus familiaris* (dog), or *Bos Taurus* (cow). We focused on chromosome 1 from position 1264279 to position 1266726, which corresponds to the entire region between the annotated human *T1R3* transcriptional start site and the nearby *GLTPD1* open reading frame (Suppl Fig. 1), because, in general, the *cis*-regulatory regions resides at upstream of transcriptional start of the genes [30]. This revealed that, while the nucleotide conservation of this entire region between human and rhesus macaque is relatively high (percent identity = 76.1%), it is low between human and mouse or rat (percent identity = 20.9% and 22.7%, respectively) (Suppl Table 1).

3.2. Identification of human T1R3 promoter region

To identify the human *T1R3* promoter, we constructed a series of