Biochemical and Biophysical Research Communications 465 (2015) 625–630

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tsukushi expression is dependent on Notch signaling and oscillated in the presomitic mesoderm during chick somitogenesis

Uzzal Kumar Acharjee [a, b], Ryu Gejima [a], M. Felemban Athary Abdulhaleem [a], M. Asrafuzzaman Riyadh [a], Hideaki Tanaka [a], Kunimasa Ohta [a, c, *]

[a] Division of Developmental Neurobiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[b] Program for Leading Graduate Schools HIGO (Health Life Science: Interdisciplinary and Glocal Oriented), Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[c] Japan Agency for Medical Research and Development (AMED), Tokyo 100-0004, Japan

ARTICLE INFO

Article history:
Received 10 August 2015
Accepted 17 August 2015
Available online 20 August 2015

Keywords:
Tsukushi
Notch signaling
Somitogenesis
Chick
Oscillation

ABSTRACT

During somitogenesis, segmentation of the body axis occurs by epithelial somites budding off from the rostral end of the unsegmented presomitic mesoderm (PSM), and its molecular regulation is achieved by a molecular oscillator and signaling molecules. Tsukushi (TSK) is a unique secreted protein and involved in diverse biological cascades in vertebrate embryos by modulating several signaling pathways at the extracellular region. However, the involvement of *TSK* in somitogenesis remains unknown. In this study, we investigated the detailed expression patterns of *TSK* at different developmental stages of a chick embryo. Chick-*TSK* (C-*TSK*) is expressed in the PSM and shows an oscillation pattern with three phases. The oscillation pattern of C-*TSK* in the PSM is similar to that of c-*Notch1* and c-*hairy1*, but not to c-*Delta1*. Our *in vitro* data showed that Notch signaling is necessary for the normal expression of C-*TSK* and that expression of C-*TSK* is an intrinsic property of the anterior PSM. These data suggest that *TSK* plays a role in chick somitogenesis.

1. Introduction

Somitogenesis is one of the most fascinating and fundamental processes of vertebrate early development by which the blueprint of the vertebral column, the "somites", form from both sides of the developing neural tube [1]. These somites are transient bilateral repeated segments of the paraxial mesoderm that differentiate into the axial skeleton, body skeletal musculature, and dermis. Segmentation of the vertebrate body axis is initiated through somitogenesis, whereby epithelial somites bud off in pairs periodically from the rostral end of the unsegmented presomitic mesoderm (PSM) [2].

Somite formation involves extensive cellular readjustments, namely, cell packing and polarization, when preparing for the required mesenchymal-to-epithelial transition [3]. It is widely accepted that this process is controlled by a molecular oscillator that drives periodic waves of gene expression caudo-rostrally through the PSM with the same periodicity as somite formation [4]. Several clock genes belonging to the Wnt, Notch, shh, or FGF pathways play roles in the process of somite formation and oscillation [5]. This periodic mechanism is repeated a specific number of times until the embryo acquires a defined species-specific final number of somites at the end of the process of axis elongation [4].

Previously, we reported that Tsukushi (TSK) is a member of the Small Leucine-Rich Proteoglycan (SLRP) family belonging to the subclass IV [6]. *TSK* binds nodal/VG1 [7], BMP4/Chordin [6], FGF8 [8], Frizzled4 [9], and Delta [10] and modulates their downstream intracellular signaling pathways, indicating multiple regulatory functions of *TSK* during early development. *TSK* is also involved in anterior commissure formation in the mouse brain [11]. *TSK*, therefore, play multiple roles as a signaling mediator at the extracellular region during many key biological events [12].

In this study, we investigated the detailed expression pattern of C-*TSK* at different developmental stages of chick somitogenesis. Using an *in situ* hybridization technique, we found that C-*TSK* is expressed in the PSM and shows an oscillation similar to that of c-*hairy1* and c-*Notch1*, but not to c-*Delta1*. Further, an *in vitro* culture assay showed that Notch signaling is necessary for the normal

* Corresponding author. Division of Developmental Neurobiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
E-mail address: ohta9203@gpo.kumamoto-u.ac.jp (K. Ohta).

http://dx.doi.org/10.1016/j.bbrc.2015.08.074
0006-291X/

expression of C-*TSK* and that the expression of C-*TSK* is an intrinsic property of the PSM. Our results suggest that *TSK* plays a role in chick somitogenesis with oscillation by interacting Notch signaling.

2. Materials and methods

2.1. Chick

Chick eggs were incubated at 38 °C in a humidified incubator. The embryos were staged according to the developmental table of Hamburger and Hamilton [13]. The stage specific (stage 7, stage 8, stage 10, stage 15, stage 18, stage 23) embryos were used for *in situ* hybridization. The newly formed somites were designated S1, S2, S3, the forming somite was designated S0, and the one somite length unsegmented PSM was designated S-1. All experiments on chick were conducted in accordance with the guidelines of the Center for Animal Resources and Development (CARD), Kumamoto University, Japan.

2.2. In situ hybridization

In situ hybridization was performed as previously described [6] to assess C-*TSK*, c-*hairy1*, c-*Notch1* and c-*Delta1* expression patterns in the presomitic mesoderm. For experiment on oscillation pattern, stage 15 (somites 25) embryos were selected by counting the somite numbers, cut the embryos into two equal halves, and complementary pairs were used for *in situ* hybridization. Sense and antisense riboprobes labeled with Digoxigenin (DIG) were synthesized from the corresponding DNA constructs [14]. A color reaction was initiated with 337.5 μg/ml 4-Nitro blue tetrazolium (NBT, Roche) and 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP, Roche) in freshly prepared Alkaline Phosphatase (AP) buffer (0.1 M Tris–HCL, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 1% Tween-20, and 0.48 mg/ml levamisole hydrochloride).

2.3. Chick embryo and explants in vitro culture

Chick embryos ranging from 15 to 20 somites were cultured in L15 medium (L-15 Leibovitz medium, Gibco) supplemented with 15% fetal calf serum (FCS) and 0.1% gentamycin (Sigma Aldrich) in a roller incubator at 37 °C. For pharmacological inhibition of signaling pathways, embryos were cultured for 9 h in 10–100 μM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, (DAPT, Calbiochem). The DAPT was diluted in culture medium from stock solutions in dimethylsulphoxide (DMSO) to maintain a final concentration of 1%. For *in vitro* culture of the explants (stage 14) containing PSM, embryos were isolated from surrounding tissues and the cultured (0–6 h) embryos were hybridized with C-*TSK* probes.

2.4. Image analysis

Embryos processed for whole mount *in situ* hybridization were photographed, using an Olympus DP70 digital camera coupled with a Leica MZFLIII stereomicroscope equipped with a DP Controller image manager (Olympus Corporation, Japan). Cryosectioned samples were photographed using a Keyence BZ-9000 fluorescence microscope (Keyence Corporation, Japan).

3. Results

3.1. C-TSK is expressed in PSM during chick somitogenesis

During chick embryogenesis, C-*TSK* is expressed in various tissues, including the limb bud, nasal pit, and PSM [7,8]. To determine *TSK* mRNA expression in detail in the PSM and newly forming somites, we examined the expression pattern of C-*TSK* in the PSM of different developmental stages of chick embryos (Fig. 1). C-*TSK* was detected beginning at stage 7, where the first somite pair originated

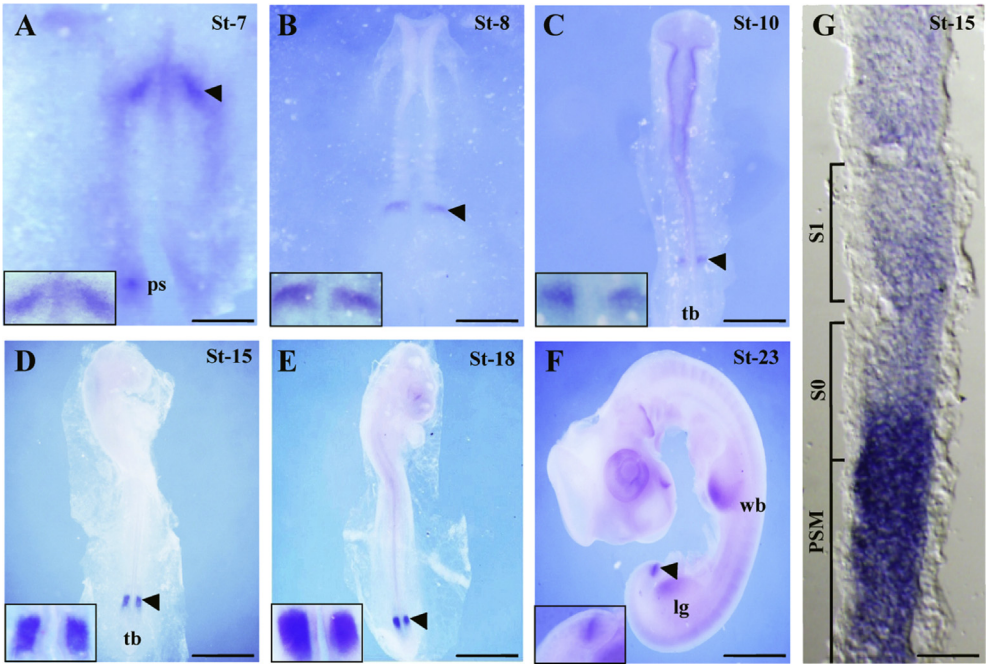

Fig. 1. Expression of C-*TSK* in the PSM at different stages of chick embryos. (A–F) Whole mount *in situ* hybridization. C-*TSK* is detected at stage 7 (A), stage 8 (B), stage 10(C), stage 15 (D), stage 18 (E), and stage 23 (F) (violet color in all stages). The black arrowheads show the PSM expressing C-*TSK*. The boxed areas are magnified views of the PSM. (G) Sagittal section of the PSM at stage 15, where C-*TSK* is expressed weakly in the caudal half of the newly formed somite 'S1', strongly from the caudal half of the forming somite 'S0' and in the unsegmented posterior PSM. ps, primitive streak; tb, tail bud; wb, wing bud; lg, leg bud. Scale bars: 150 μm in A; 300 μm in B; 400 μm in C–E; 2 mm in F; 50 μm in G. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)