Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 359 (2007) 959–964
BBRC
www.elsevier.com/locate/ybbrc

Bowline mediates association of the transcriptional corepressor XGrg-4 with Tbx6 during somitogenesis in *Xenopus*

Akiko Kondow [a,1,2], Keisuke Hitachi [a,2], Koji Okabayashi [b], Nobuhiro Hayashi [c], Makoto Asashima [a,b,d,*]

[a] *Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*
[b] *ICORP Organ Regeneration Project, Japan Science and Technology Agency (JST), 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*
[c] *Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan*
[d] *Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan*

Received 30 May 2007
Available online 11 June 2007

Abstract

Prior to the somite segmentation, the cells in the anterior presomitic mesoderm (PSM) express a set of genes that is required for defining the segmental border and polarity of the prospective somite. However, little is known how the expression of these genes is repressed upon segmentation. Here we report that Bowline, an associate protein of the transcriptional corepressor XGrg-4, repressed Tbx6 dependent transcription of *Thylacine1* by mediating interaction of Tbx6 with XGrg-4 in *Xenopus laevis*. In *bowline*-deficient embryos, segmental border formation was disturbed, and expression of *Thylacine1*, *X-Delta-2*, and *bowline* expanded anteriorly. Tbx6-dependent transcription of *Thylacine1* was suppressed by Bowline, together with XGrg-4. We also found that Bowline mediated the interaction of Tbx6 and XGrg-4. Based on our findings, we conclude that a part of the transcriptional repression at the anterior end of the PSM is caused by Bowline mediated transcriptional repression of Tbx6-dependent gene expression in *X. laevis*.

Keywords: Presomitic mesoderm; Thylacine1; Xenopus laevis; Tbx6; Groucho/TLE; XGrg-4; Ripply1

The bodies of vertebrates are subdivided into segments called somites along the anteroposterior axis. Somites give rise to vertebrae, ribs, and skeletal muscles, and provide a design for other segmental patterns, such as those of the vascular and peripheral nervous systems.

Each somite segregates from the anterior end of the presomitic mesoderm (PSM) one at a time, at a species-specific constant rate. A prospective border is defined prior to the segmentation in the anterior PSM. In mouse, borders form at the boundary of the Notch active domain and the expression domain of a basic helix–loop–helix (bHLH) transcription factor, Mesp2 in the PSM [1]. Mesp2 defines the segmental border by suppressing Notch signaling via activation of *L-fng* [1]. In *Xenopus laevis*, a homologue of *Mesp2, Thylacine 1* (*Thy1*), is expressed in the rostral halves of the prospective somites, as is for the Notch ligand *X-Delta-2,* while *X-hairy-2A* is expressed in the caudal halves of the prospective somites [2,3]. As the cells segregate to form a new somite, the transcription of those genes is subsequently repressed; thus, their expression region is confined to the PSM posterior to the newly formed somite. However, the molecular mechanisms underlying the repression of the genes are not well understood.

* Corresponding author. Address: Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan. Fax: +81 3 5454 4330.
E-mail address: asashi@bio.c.u-tokyo.ac.jp (M. Asashima).
[1] Present address: Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan.
[2] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.05.211

Recently, Ripply1 in zebrafish was reported to be required for the gene repression process. In *ripply1*-deficient zebrafish, gene expressions characteristic to the anterior PSM do not terminate properly [4]. Ripply1 belongs to a novel Ripply–Bowline–Ledgerline (RBL) proteins implicated in somitogenesis [4–9]. Ripply1 and Bowline, an RBL protein in *X. laevis*, were shown to associate with the transcriptional corepressor Groucho/TLE, and repress target gene expression in the anterior PSM [4,5]; for this reason they have been proposed to function in transcriptional repression. However, no DNA binding motif has been found for the RBL proteins, so it is not known how they determine their target genes.

In this study we investigated the mechanism of Bowline-mediated gene repression to understand how a set of genes is repressed synchronously during the formation of a new somite. We assessed requirement of Bowline in transcriptional repression in the anterior PSM by (1) the knockdown experiment of *bowline* in vivo, and (2) the promoter analysis of a Bowline target gene *Thy1* in vitro. Furthermore, a novel Bowline-interacting protein Tbx6 was identified, and its role in Bowline-mediated transcriptional repression was investigated.

Materials and methods

Embryo manipulation and morpholino oligomers (MOs). *Xenopus laevis* embryos were collected according to standard procedures, as previously described [5]. Antisense MOs for *X. laevis bowline*, *bowline2* and Control MO (CoMO) were obtained from Gene Tools: bowline MO, 5′-AGTG TCTCCACGAGTTTCTTTGCAC-3′; bowline2 MO, 5′-CGATTATC TCTGCACGGTTCTTTGC-3′; CoMO, 5′-CCTCTTACCTCAGTTAC AATTTATA-3′.

RNA synthesis. Capped mRNAs were synthesized with the mMessage mMachine kit (Ambion). To generate synthetic RNA coding for Bowline or Bowline2 fused with myc tag at their C-termini in vitro, the coding region of *bowline* or *bowline2* cDNA was subcloned into pCS2+MT vector (pCS2+bowline-myc, pCS2+bowline2-myc). For Bowline-myc mutant lacking the Ripply/BDLC domain, the DNA sequence corresponding to amino acid sequence 93–128 of Bowline was deleted from pCS2+bowline-myc. For Tbx6 fused to HA tag at the C-terminus, the coding region of Tbx6 cDNA was subcloned into HA-tagged pCS2+ vector [10]. Templates for Bowline and myc-tagged XGrg-4 were as previously described [5].

Whole-mount in situ hybridization and histological analysis. Whole-mount in situ hybridization was performed as previously described [5]. The templates for *Thy1*, *X-Delta-1,* and *X-Delta-2* have been described previously [3,6,11]. The template for *X-hairy-2* (Accession No. AF139914) was kindly provided by Dr. Y. Sasai (RIKEN). Probes against *bowline* intron were developed against 1st and 3rd introns of the *bowline* gene (Accession Nos. AB308385, AB308386).

For histological analysis, embryos were fixed in MEMFA for 1 h at room temperature or overnight at 4 °C. Embryos were embedded in paraffin, and parasagittal sections of 6–10 μm thickness were stained with hematoxylin.

Cell lines, transfection, and luciferase assays. The reporter construct pGL4.2Thy1 was generated by inserting the SalI/BglII fragment of pSP72-Thy1 corresponding to the 5′ adjoining sequence of the *Thy1* open reading frame (Accession No. EF205628) into the XhoI/BglII site of pGL4.20 (Promega) [12]. Expression constructs for Tbx6, Tbx6-VP16, XotchΔE, XGrg-4, and Bowline were as previously reported [5,10,13,14]. To disrupt the WRPW sequence of Bowline, the DNA sequence corresponding to WRPW (amino acids 58–61) was removed (BowlineΔWRPW), or Arg at amino acid 59 was replaced by Glu (BowlineWEPW). For Bowline mutant lacking the Ripply/BDLC domain (BowlineΔBDLC), the DNA sequence corresponding to amino acid 93–128 was deleted. pSP72-Thy1 was kindly provided by Dr. C. Kintner (The Salk Institute, USA).

COS7 cells maintained in DMEM supplemented with 10% fetal bovine serum and 1× penicillin–streptomycin were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen) in 24-well plates. The total amount of transfected DNA was kept constant by supplementing with the construct in which β-gal gene is subcloned into pCS2+ vector (pCS2-β-gal). Total 425 ng of DNA was transfected (20 ng of reporter construct, 300 ng of expression vector coding for Tbx6-VP16, 150 ng for Tbx6 and XotchΔE, 50 ng for Bowline, BowlineΔBDLC, BowlineΔWRPW, BowlineWEPW, or XGrg-4). Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega). As a reference to normalize transfection efficiency, 5 ng of pGL4.74 was cotransfected in all experiment. The experiments were performed at least twice in triplicate for each assay and representative data are shown.

Immunoprecipitation and Western blot analysis. Immunoprecipitation was performed as described previously with modifications [5]. One blastomere of two-cell stage embryos was injected with the following RNAs in combination: *XGrg-4-myc, bowline-myc, bowline, bowlineΔBDLC-myc*, *Tbx6-HA*, and β-gal. Embryos were homogenized at stage 10–11. One-hundred embryos were used for each immunoprecipitation and 0.9% of each lysate was used as input. The extract was incubated with anti-myc antibody (9E10; Sigma) coupled to Protein G beads (Amersham). Western blot analysis was performed using rabbit anti-myc antibody (562; MBL) or Anti-HA-peroxidase conjugated High Affinity clone 3F10 (Roche).

For confirmation of inhibition of translation by antisense MO, protein was isolated from a pool of 5–10 embryos at stage 10.5–12, and protein corresponding to a single embryo was loaded for each electrophoresis. Western blot analysis was performed using anti-myc antibody (9E10; Sigma) or monoclonal anti-α-tubulin antibody (Santa Cruz).

Results

Bowline is required for somite segmentation

To elucidate the function of Bowline in somite segmentation in *X. laevis*, we blocked translation of *bowline* mRNA using an antisense MO against the 5′ untranslated region (UTR) of the *bowline* gene. To block translation of the pseudo allele of *bowline* simultaneously, we identified an expressed sequence tag (EST) clone with high homology to *bowline* (Accession No. DR718559, 80.4% amino acid identity) and determined its 5′ UTR sequence using the 5′ rapid amplification of cDNA ends (RACE) method (Accession No. AB300353). We named it *bowline2*. Using Western blot hybridization, we confirmed that antisense MOs against either the *bowline* or *bowline2* genes specifically blocked the translation of the myc-tag fusion products of the respective genes (Fig. 1A).

In embryos injected with antisense MOs against *bowline* and *bowline2* (BlnMO), somitic cells were randomly scattered and failed to elongate, in contrast with embryos injected with CoMO in which the extended somitic cells were aligned parallel to the body axis (Fig. 1B), indicating the requirement of Bowline protein in normal somitic border formation.

We next examined the influence of *bowline*-knockdown on expression of genes implicated in somite segmentation, namely *Thy1*, *X-Delta-1*, *X-Delta-2*, *X-hairy2*, and *bowline*. In embryos injected with BlnMO, additional bands of *Thy1* expression were observed at region rostral to the intact