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Molecular characterization of tripartite motif protein 25 (TRIM25) involved in ERα-mediated transcription in the Korean rose bitterling *Rhodeus uyekii*

Hee Jeong Kong [a,*], Ye Ji Lee [a], Jihye Shin [b], Hyun Kook Cho [c], Woo-Jin Kim [a], Hyung Soo Kim [a], JaeHun Cheong [c], Young Chang Sohn [b], Sang-Jun Lee [a], Bong-Seok Kim [a]

[a] *Biotechnology Research Division, National Fisheries Research and Development Institute, Busan 619-705, Republic of Korea*
[b] *Department of Marine Molecular Biotechnology, Gangneung–Wonju National University, Gangneung 210-702, Republic of Korea*
[c] *Department of Molecular Biology, Pusan National University, Busan 609-735, Republic of Korea*

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ABSTRACT

Tripartite motif-containing 25 (TRIM25), also known as estrogen-responsive finger protein (EFP), plays an essential role in cell proliferation and innate immunity. In the present study, we isolated and characterized the TRIM25 cDNA of the Korean rose bitterling *Rhodeus uyekii*, designated RuTRIM25. It encodes an open reading frame of 669 amino acids containing an N-terminal RBCC motif composed of a RING domain, two B boxes, and a coiled-coil domain and a C-terminal B30.2 (PRY/SPRY) domain. RuTRIM25 shows strong homology (79.7%) to zebrafish TRIM25 and shared 32.4–28.8% homology with TRIM25 from other species, including mammals. RuTRIM25 mRNA was expressed ubiquitously. It was highly expressed in the ovary, spleen, and liver and moderately in the stomach and intestine of normal Korean rose bitterling. The intracellular localization of RuTRIM25 in HEK293T cells was diffusely localized in the cytoplasm and its RING domain deletion mutant (RuTRIM25ΔR) was detected diffusely with some aggregates in the cytoplasm. RuTRIM25, but not RuTRIM25ΔR, is ubiquitinated *in vivo*. Ectopic expression of RuTRIM25 synergistically activated the estrogen receptor (ER)-mediated luciferase reporter activity in a dose-dependent manner in HEK293T cells. Together, these results suggest that the RuTRIM25 regulates the ER-mediated transcription in fish similarly to its mammalian counterpart.

1. Introduction

The tripartite motif-containing (TRIM) family is characterized by the presence of an N-terminal RBCC motif consisting of a RING domain, one or two zinc-binding motif B boxes, and a coiled-coil region (Torok and Etkin, 2001; Meroni and Diez-Roux, 2005). The RBCC motif is usually followed by either one or two C-terminal domains, of which there are 10 distinct motifs including B30.2 (PRY/SPRY) (the most common in humans), COS (C-terminal subgroup one signature), fibronectin type 3, and immunoglobulin (Short and Cox, 2006; Ozato et al., 2008). The numbers of TRIM family members vary between species; more than 60 members are found in humans and mice, 240 in zebrafish, and 10–20 in worms and flies (Ozato et al., 2008; Sardiello et al., 2008; Van der Aa et al., 2009). The tripartite motif-containing (TRIM) family is one of the subfamilies of the RING finger E3 ubiquitin ligase family (Joazeiro and Weissman, 2000; Meroni and Diez-Roux, 2005). This family is involved in a variety of cellular functions including development, cell proliferation, differentiation, oncogenesis, apoptosis, and innate immunity.

Human TRIM25 was originally identified as estrogen-responsive finger protein (EFP) by genomic binding site cloning using a recombinant estrogen receptor (ER) protein and has an estrogen response element (ERE) consensus sequence at the regulatory region (Inoue et al., 1993). The expression of TRIM25 was predominantly in estrogen target tissues, including mammary glands, uteri, and osteoblasts and in immune-related tissue, such as the spleen and thyroid gland (Orimo et al., 1995; Inoue et al., 1999; Reymond et al., 2001; Shimada et al., 2004). It was induced by estrogen treatment in the MCF-7 human breast carcinoma cell line and type I interferon in macrophages and dendritic cells (Ikeda et al., 1997; Rajsbaum et al., 2008).

TRIM25 regulates various cellular processes through E3 Ub ligase activity. TRIM25 targets the negative cell cycle regulator, 14-3-3σ, for proteolysis and promotes breast tumor growth (Urano et al., 2002). Ligand-dependent transcription of ERα is regulated by TRIM25, which is dependent on the RING domain (Nakajima et al., 2007). TRIM25 activates the viral RNA receptor retinoic acid-inducible gene (RIG)-I-mediated antiviral innate immune response (Gack et al., 2007; Gack et al., 2008). In addition, TRIM25 functions as an E3 ligase for interferon-stimulated protein 15 kDa (ISG15)

* Corresponding author at: Biotechnology Research Division, National Fisheries Research and Development Institute, 408-1 Sirang-ri, Gijang-up, Gijang-gun, Busan 619-705, Republic of Korea. Tel.: +82 51 720 2453; fax: +82 51 720 2456.
E-mail addresses: konghj@nfrdi.go.kr, heejkong@hotmail.com (H.J. Kong).

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conjugation to 14-3-3σ, suggesting that TRIM25 is a common component in the ubiquitin and ISG15 conjugation pathway (Nakasato et al., 2006; Zou and Zhang, 2006; Zou et al., 2007).

The first fish TRIM genes were bloodthirsty from Antarctic rockcod, blackfin icefish and zebrafish, which play a role for erthropoiesis in zebrafish (Yergeau et al., 2005). Atlantic cod bloodthirsty containing B30.2 (PRY/SPRY) domain was identified as an immune-related gene (Furnes and Robertsen, 2010). A large number of TRIM family members have been also isolated in pufferfish, rainbow trout, and zebrafish (Sardiello et al., 2008; Du Pasquier, 2009; van der Aa et al., 2009). Zebrafish TRIM3a might play an important role in brain development and function as a RING finger E3 ubiquitin ligase (Zhang et al., 2012). Several fish E3s also have been identified in rainbow trout: MuRF involved in muscle degradation and atrogin-1/FBXO32 and FBXO25 responsible to feed deprivation (Cleveland and Evenhuis, 2010; Wang et al., 2011).

Korean rose bitterling *Rhodeus uyekii* is an endemic Korean fish belonging to the Acheilognathinae subfamily of the Cyprinidae family. It is distributed in rivers that empty into the Yellow Sea and Southern Sea in Korea. Recently, this species has been considered as a candidate for development as an ornamental fish in Korea (Kang et al., 2005). In this study, we report the identification and characterization of TRIM25 of the Korean rose bitterling *R. uyekii* as a homolog of other TRIM25.

2. Materials and methods

2.1. Sequence analysis

Expressed sequence tag (EST) clones were isolated from *R. uyekii* total cDNA libraries using a plasmid miniprep kit (Qiagen), and then sequenced using T3 reverse primers (Promega) on an ABI3730xl automatic sequencer (Applied Biosystems, Inc.). The cDNA sequence was annotated using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/) in GenBank.

2.2. Multiple sequence alignment and phylogenetic analysis

A multiple sequence alignment was created using ClustalW 2.1. Multiple sequence alignments were used to assess similarities among the aligned sequences (http://www.genome.jp/tools/clustalw/). A phylogenetic tree based on the deduced amino acid sequences was constructed using the neighbor-joining algorithm, and the reliability of the branching was tested using bootstrap resampling with 1000 pseudo-replicates.

2.3. Fish maintenance and tissue samples

Individuals of *R. uyekii* were collected from the Yangchun River, Uiryung-gun, Gyungnam, Republic of Korea. The fish were maintained at the National Fisheries Research and Development Institute (NFRDI) in Busan, Republic of Korea. The adults were kept in 40-l glass aquaria at a density of around 20 fish per aquarium. The water was renewed weekly; the temperature in the rearing tanks was maintained at 20 ± 1 °C. The room was maintained on a 12-h/12-h light–dark cycle. The adults were fed TetraBits (Tetra) and frozen bloodworms (Advanced Hatchery Technology) two times a day. For RNA extraction, the tissues from three fish were removed, immediately frozen in liquid nitrogen, and stored at −80 °C before use.

2.4. Quantitative real-time RT-PCR

Total RNA was prepared from tissues using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. The total RNA concentration was quantitatively determined, and 1 μg of total RNA was used for reverse transcription. The first-strand cDNA was synthesized using an Advantage® RT-for-PCR kit (BD Sciences). Quantitative real-time PCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Roche) and the following forward and reverse primers: for RuTRIM25, RuTRIM25-RT-F (5′-GAT CCA GGA ATC CCC CTA AA-3′) and RuTRIM25-RT-R (5′-ATC CGC TTG TGA GCT GTT CT-3′), for Ru-b-Actin (GenBank accession no. JQ279058), Ru-b-Actin-F (5′-GAT TCG CTG GAG ATG ATG CT-3′) and Ru-b-Actin-R (5′-ATA CCG TGC TCA ATG GGG TA-3′). Following an initial 10-min Taq activation step at 95 °C, LightCycler PCR was performed for 40 cycles using the following cycling conditions: 95 °C for 10 s, 57 °C for 5 s, 72 °C for 30 s, and fluorescence reading. Transcript levels were quantified by expression relative to the *b*-Actin transcript level.

2.5. Construction of the expression plasmid

Amplification of the open reading frame (ORF) of the full length RuTRIM25 (1–636 aa) and the RING deletion mutant (RuTRIM25ΔR; 58–636 aa) was carried out using the vent DNA polymerase (New England Biolab). The primers were designed so that the amplified DNA would contain BamHI and EcoRV restriction endonuclease sites at its 5′ and 3′ ends, respectively. The primer sequences were as follows: forward, 5′-GCG GAT CCA TGG CGG AAA ATA TGT CT-3′ for full length and 5′-GCC GGA TCC ATG GCT TTC AGC AGC AAA CCA-3′ for the RING deletion mutant; reverse, 5′-CGG ATA TCT CAG TTG CTG AGC TTG CA-3′. The amplified cDNA fragments were inserted into BglII and SmaI restriction endonuclease sites of the pEGFP-C1 vector (Clontech) for mammalian expression. The sequences of the constructs were confirmed by sequencing.

2.6. Cell culture

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL) and 1% (v/v) antibiotic–antimycotic (AA; GIBCO BRL) at 37 °C in a humid 5% CO_2 atmosphere.

2.7. Confocal microscopy

Cells were seeded on glass coverslips in six-well plate and transiently transfected with 500 ng of GFP/RuTRIM25, GFP/RuTRIM25 deletion mutant, or empty vector (GFP only) using PolyFect (Qiagen). Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4), mounted using VECTASHIELD® with DAPI (Vector Labs), and viewed using an LSM700 laser scanning confocal microscope (Carl Zeiss). The localization of GFP-fusion protein or control GFP protein in the cells was demonstrated by direct fluorescence.

2.8. SDS-PAGE and immunoblot analysis

The cells were prepared by washing with cold-PBS and lysed. The protein concentration was determined using the BSA as a standard and Bradford reagent (Bio-Rad). Equal amounts of proteins were loaded and separated by SDS-PAGE and the gels were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). For Western blotting, the membranes were incubated with anti-HA (Roche), anti-GFP (Santa Cruz Biotechnology), and anti-actin (Sigma) in TBST supplemented with 3% non-fat dry skim milk overnight at 4 °C. After washing three times with cold TBST, the blotted membranes were incubated with peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at room temperature. After washing three times with cold TBST, the proteins were visualized by the enhanced chemiluminescent development reagent (Amersham Pharmacia Biotech).