Biochemical and Biophysical Research Communications 497 (2018) 597–604

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Ess2 bridges transcriptional regulators and spliceosomal complexes via distinct interacting domains

Ichiro Takada [a, *], Megumi Tsuchiya [b], Kaori Yanaka [c], Shinya Hidano [d], Sayuri Takahashi [e], Takashi Kobayashi [d], Hidesato Ogawa [b], Sinichi Nakagawa [c], Makoto Makishima [a, **]

[a] Division of Biochemistry, Department of Biomedical Sciences, School of Medicine, Nihon University, Itabashi-ku, Tokyo 173-8610, Japan
[b] Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita 565-0871, Japan
[c] RNA Biology Laboratory, RIKEN Advanced Research Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[d] Department of Infectious Diseases Control, Faculty of Medicine, Oita University, Oita 879-5593, Japan
[e] Department of Urology, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

ARTICLE INFO

Article history:
Received 31 January 2018
Accepted 12 February 2018
Available online 16 February 2018

Keywords:
Transcription
Nuclear receptor
Splicing
lncRNA

ABSTRACT

Transcription and pre-mRNA splicing are complex, coupled processes that involve transcriptional co-regulators. Ess2 (also termed Dgcr14) is a nuclear protein that enhances the transcriptional activity of retinoic acid receptor-related orphan receptor gamma/gamma-t (Rorγ/γt). Ess2 is also a component of the spliceosomal C complex (containing U2, U5 and U6 snRNAs). However, the domains in Ess2 that function in splicing and transcription have not been identified.

To elucidate the roles of Ess2 in splicing and transcription, we performed RNA immunoprecipitation (RIP) assays to detect Ess2-interacting snRNAs. We found that Ess2 associated with U6 snRNA as well as U1 and U4 snRNAs. Experiments using Ess2 deletion mutants showed that a C-terminus deletion mutant of Ess2 (1-399 a. a.) lost its ability to associate with snRNAs, whereas the N-terminus domain of Ess2 (1-200 a. a.) associated with Rorγ/γt, but not with snRNAs.

Interestingly, experiments using anti-ROR common antibody showed that Rors also associated with U4 and U6 snRNAs. *Ess2* knockdown in a T cell hybridoma (68-41 cells) abrogated the interaction between spliceosomes and Rors. An Ess2-dependent association was also found between an lncRNA (*Rmrp*) and Rors. We thus propose that Ess2 associates with both transcriptional factors and spliceosomal complexes and modulates splicing reactions coupled with transcription factors.

1. Introduction

Eukaryotic transcription is a complex reaction involving a number of proteins as well as long non-coding RNAs (lncRNAs) [1–3]. When transcription factors (TFs) bind to target DNA sequences, TFs change the association of chromatin remodeling factors and epigenetic modifiers, processes that are essential for transcriptional regulation. Over the past 2 decades, transcriptional co-activators and co-repressors have been identified and their functions defined [4].

We identified Ess-2 splicing factor homolog (Ess2; also named DiGeorge-syndrome critical region 14 (Dgcr14)) as a transcriptional co-activator for retinoic acid receptor-related orphan nuclear receptor gamma/gamma-t (Rorγ/γt). It was first observed during the differentiation of pro-inflammatory T helper 17 (T^H17) cells through its interaction with the Rorγ/γt-Baz1b-Rsk2 complex [5]. Ess2 is a nuclear protein with a coiled-coil domain [6] that is conserved from yeasts to humans. The *ESS2/DGCR14* gene is located at 22q11.2, and its deletion is associated with DiGeorge syndrome [6,7]. The syndrome is characterized by varying defects of the heart, thymus and parathyroid glands [8]. Ess2 associates with other nuclear proteins [9] or the spliceosomal C complex, containing U2, U5 and U6 snRNAs [10]. However, the molecular mechanism by which Ess2 protein regulates transcription is still unclear. Although N-terminal or C-terminal deletion mutants of Ess2 reduced mRNA expression levels of *Il17a* [5], no functional motifs related to transcription or splicing have been identified in Ess2.

Rorγ/γt belongs to a nuclear receptor (NR) family of

* Corresponding author.
** Corresponding author.
E-mail addresses: takada.ichiro@nihon-u.ac.jp (I. Takada), makishima.makoto@nihon-u.ac.jp (M. Makishima).

https://doi.org/10.1016/j.bbrc.2018.02.110
0006-291X/

transcription factors. It is specifically expressed in T cell compartments [11,12]. Most NRs are regulated by lipid-soluble ligands and some synthetic compounds for Rorγ/γt have been identified [13]. While NRs integrate their ligand signals into the histone code and chromatin remodeling [14], studies have demonstrated that co-regulators of NRs also associate with spliceosomal components and control their activities [15–17]. Furthermore, a recent study using siRNA library screening identified multiple DNA binding factors, including NRs and chromatin remodelers, as regulators of alternative splicing [18]. These studies suggest that there is a strong link between transcriptional activation and pre-mRNA splicing. Currently, the precise molecular mechanisms that control the cross-talk between the two reactions are not well understood.

In this report, we performed RNA immunoprecipitation (RIP) assays using anti-ESS2 antibodies and Ess2 deletion mutants to elucidate functional domains of Ess2 that interact with the spliceosome and transcriptional factors. We found that a transcription factor (Ror) and snRNAs independently bind discrete regions of Ess2, suggesting that Ess2 may bridge transcriptional regulations with splicing reactions.

2. Materials and methods

2.1. Plasmids

To generate expression vectors for Ess2-EGFP, we amplified deletion mutants of mouse *Ess2* cDNAs (1-202 a. a., 1-307 a. a., 1-399 a. a., 101-480 a. a., 203-399 a. a., 308-480 a. a. and 442-480 a. a.) from an *Ess2* expression vector [5] and EGFP from pEGFP-C1 (Clontech) by RT-PCR. Amplified fragments were ligated into pcDNA3 (Invitrogen) by EcoRI-XhoI (*Ess2* cDNAs) and XhoI-XbaI (EGFP). Expression vectors of GST-*Ess2* mutants were ligated into pGEX4T-1 (GE Healthcare, Inc.) at the EcoRI-XhoI site. The Rorγt expression vector was a gift from Prof. A Yoshimura (Keio University).

2.2. Cell culture

HEK293 (human embryonic kidney 293) and U2OS (U2 human osteosarcoma) cells were cultured in DMEM high glucose medium (Wako Pure Chemical Industries, Ltd.) supplemented with 10% FBS (Thermo Fisher Scientific), 50 U L^{-1} penicillin and 50 μg L^{-1} streptomycin (Nacalai Tesque Inc.). After transfection of each vector into HEK293 cells, the culture was subjected to G418 selection (10 μg/mL) for 2–3 weeks and EGFP-positive cells were sorted by flow cytometry (MoFlo XDP, Beckman Coulter) 3 times.

Mouse T cell hybridoma 68-41 cells were maintained in RPMI 1640 (Wako Pure Chemical Industries, Ltd.) supplemented with 10% FBS, 50 U L^{-1} penicillin, 50 μg L^{-1} streptomycin and 100 nM non-essential amino acids (Thermo Fisher Scientific). 68-41 cells expressing each shRNA (shluc and shEs) were generated as previously reported [5].

2.3. Immunological reagents

The following antibodies were used: anti-ESS2 (rabbit polyclonal sc-86411, Santa Cruz), anti-GFP (Z2593N, Takara Bio), anti-ROR common (PP-H3925, Perseus Proteomics), anti-SART1 (AB HPA031188, Atlas Antibodies), anti-BRR2 (A303-454A anti-SNRNP200, Bethyl Laboratories, Inc.), anti-CDC40 (ab175924, Abcam) and anti-β-actin (A1978, Sigma).

Mouse monoclonal antibodies against Ess2 were raised against a GST-tagged *Ess2* peptide (1-400 a. a.) and affinity purified. Using immunized mice, hybridoma cells were generated and confirmed. Anti-ESS2 antibody-producing hybridomas were provided by RCB4685 (RIKEN).

2.4. Fluorescent immunostaining

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing with 0.1% Tween-20 in PBS (PBS-T), cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and washed 3 times with PBS-T. The cells were then blocked with Blocking One solution (03953-95; Nacalai Tesque) for 30 min at 4 °C, washed once with PBS-T, and incubated with each antibody in Can Get Signal Immuno-stain solution A (NKB-501; Toyobo, Osaka, Japan) for 2 h, followed by 3 washes with PBS-T and then incubated with Alexa488-conjugated anti-rabbit IgG antibody and Alexa594-conjugated anti-mouse IgG antibody for 1 h. After 3 washes with PBS-T, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and subjected to fluorescence microscopy [19]. For observation, an oil-immersion objective lens (PLAPON60xOSC/NA1.40; Olympus) was used on the DeltaVision microscope system (GE Healthcare Life Sciences). The images were analyzed by using SoftWoRx 5.5 software (GE Healthcare Life Science).

2.5. RNA immunoprecipitation assays

Cells were washed with PBS and lysed for 10 min on ice using RIP buffer [20 mM HEPES (pH 8.0), 150 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5% NP-40] supplemented with RNaseOUT (Thermo Fisher Scientific) and protease inhibitor cocktail (Calbiochem). The soluble fraction was recovered after centrifugation at 18,000×*G* for 10 min at 4 °C, and subsequently used for immunoprecipitation analysis.

For immunoprecipitation, each extract (1-3 × 10^5 cells) was treated with the indicated antibody (diluted 1:200 in RIP buffer) and rotated for 1 h at 4 °C. Then, samples were incubated with Protein G-coated Dynabeads for immunoprecipitation (Thermo Fisher Scientific) for 1 h at 4 °C. The samples were washed with RIP buffer 4 or 5 times and subjected to RNA isolation with TRIzol (Thermo Fisher Scientific). cDNAs were synthesized with PrimeScript (Takara). RIP RNA fractions were normalized to the corresponding RNA input fractions. For quantitative PCR (qPCR), the ABI PRISM7000 (Thermo Fisher Scientific) was used with the Light Cycler SYBR Green I Master Mix (Takara). The relative quantitation value is expressed as $2^{-\Delta Ct}$, where ΔCt is the difference between the mean Ct values of triplicates of the sample. PCR primers are listed in the Supplementary Table.

2.6. Western blotting

Proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) for immunodetection using an electrophoretic wet transfer system (Bio-Rad Laboratories, Inc.). After transfer, membranes were blocked with PBS-T with 2.5% skim milk for 1 h at room temperature and incubated with a specific primary antibody (indicated above, diluted 1:1000 in 1.5% BSA/PBS-T) overnight at 4 °C. After washing in PBS-T, membranes were incubated with HRP-coupled secondary antibodies (DAKO) indicated above, 1:7000 in 0.75% BSA in PBS-T, for 30 min at room temperature. After 3 washes, membranes were developed by ECL Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer's instructions.

2.7. GST pull-down assays

Regions of Ess2 (1-200 a. a. and 207-480 a. a.) that were cloned into pGEX4T-1 were expressed as GST fusion proteins in *E. coli*. We