Biochimica et Biophysica Acta 1859 (2016) 964–974

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Transcriptional control of transglutaminase 2 expression in mouse apoptotic thymocytes

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ARTICLE INFO

Article history:
Received 23 February 2016
Received in revised form 20 May 2016
Accepted 31 May 2016
Available online 02 June 2016

Keywords:
T cells
Apoptosis
Gene regulation
Enhancer RNA
Transglutaminase 2
ChIP-seq

ABSTRACT

Transglutaminase 2 (TGM2) is a ubiquitously expressed multifunctional protein, which participates in various biological processes including thymocyte apoptosis. As a result, the transcriptional regulation of the gene is complex and must depend on the cell type. Previous studies from our laboratory have shown that in dying thymocytes the expression of *Tgm2* is induced by external signals derived from engulfing macrophages, such as retinoids, transforming growth factor (TGF)-β and adenosine, the latter triggering the adenylate cyclase signaling pathway. The existence of TGF-β and retinoid responsive elements in the promoter region of *Tgm2* has already been reported, but the intergenic regulatory elements participating in the regulation of *Tgm2* have not yet been identified. Here we used publicly available results from DNase I hypersensitivity analysis followed by deep sequencing and chromatin immunoprecipitation followed by deep sequencing against CCCTC-binding factor (CTCF), H3K4me3, H3K4me1 and H3K27ac to map a putative regulatory element set for *Tgm2* in thymocytes. By measuring eRNA expressions of these putative enhancers in retinoid, rTGF-β or dibutiryl cAMP-exposed thymocytes we determined which of them are functional. By applying ChIP-qPCR against SMAD4, retinoic acid receptor, retinoid X receptor, cAMP response element binding protein, P300 and H3K27ac under the same conditions, we identified two enhancers of *Tgm2*, which seem to act as integrators of the TGF-β, retinoid and adenylate cyclase signaling pathways in dying thymocytes. Our study describes a novel strategy to identify and characterize the signal-specific functional enhancer set of a gene by integrating genome-wide datasets and measuring the production of enhancer specific RNA molecules.

1. Introduction

Transglutaminases [1] are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ–carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in certain proteins. The reaction results in post-translational modification of proteins by establishing ε–(γ–glutamyl)lysine cross-linkages and/or covalent incorporation of mono- or polyamines and into proteins. Transglutaminase 2 (TGM2) is very unique in the transglutaminase family, because besides being a transglutaminase it also possesses GTPase, protein disulphide isomerase and protein kinase enzymatic activities. In addition, TGM2 can also function in various biological settings as a protein/protein interaction partner [2]. TGM2 has been known for a long time to be associated with the *in vivo* apoptosis program of various cell types including T cells [3]. TGM2 expression is induced in thymocytes dying *in vivo* following exposure to various apoptotic signals [4], and TGM2 also appears in the dying T lymphocytes of HIV-infected individuals [5]. While, however, TGM2 is strongly induced in dying thymocytes *in vivo*, no induction of TGM2 was observed, when thymocytes were induced to die by the same stimuli *in vitro* [6] indicating that signals arriving from the tissue environment contribute to the *in vivo* induction of the enzyme in apoptotic thymocytes. Later studies from our laboratory have demonstrated that in dying thymocytes the expression of TGM2 is induced by signals derived from engulfing

Abbreviations: Atf1, activating transcription factor 1; CBP, CREB binding protein; CCTF, CCCTC-binding factor; CEBP, CCAAT/enhancer-binding protein; ChIP-seq, chromatin immunoprecipitation followed by deep sequencing; 9cRA, 9-cis retinoic acid; Crem, cAMP response element modulator; CREB, cAMP response element binding protein; CRTC, CREB-regulated transcription coactivator; DNase-seq, DNase I hypersensitivity analysis followed by deep sequencing; eRNA, enhancer RNA; IGV, Integrative Genomics Viewer; RAR, retinoic acid receptor; RNAPII, RNA polymerase II; RNA-seq, RNA sequencing; RXR, retinoid X receptor; TGF-β, transforming growth factor β; TGM2, transglutaminase 2; TSS, transcription start site.

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http://dx.doi.org/10.1016/j.bbagrm.2016.05.011
1874-9399/

macrophages, such as retinoids [7], transforming growth factor (TGF)-β [8] and adenosine, the latter triggering the adenylate cyclase signaling pathway [9].

Due to its ubiquitous appearance and multiple functions, the expression of the *Tgm2* gene must be tightly controlled. The *in vivo* appearance of TGM2 protein in dying thymocytes seems to be regulated at the level of transcription, since in mice, which carry the beta-galactosidase reporter gene under the control of a 3.8 kilobase fragment of the *Tgm2* promoter, the beta-galactosidase expression showed strong correlation with the endogenous TGM2 expression [6]. Other studies also indicate that the complex regulation of TGM2 expression is mediated at the level of transcription. Thus research has led to the identification of functional retinoid [10] and TGF-β [11] response elements in the promoter region of the gene. In addition, response elements for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [12] and the hypoxia-inducible factor (HIF) [13] have been also identified in the core promoter. The limitation of these studies was that they could focus only on the promoter element at that time.

Recent advances in sequencing technologies allow genome-wide mapping of all the intergenic regulatory elements including enhancers. The combination of the DNase I hypersensitivity analysis followed by deep sequencing (DNase-seq) and chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) datasets proved to be useful in identifying and characterizing the chromatin signatures of enhancer elements [14]. These include enrichment of histone 3 lysine 4 monomethylation (H3K4me1) as compared to H3K4me3, which peaks at promoter elements [15]. However, enhancers exhibiting these modifications are not necessary functional, because most of the open regulatory elements are enriched for at least one of these markers. Functional enhancers are rather associated with histone 3 acetylated at lysine 27 (H3K27ac) [16], with the transcription co-regulators P300 or CREB binding protein (CBP) [17], and with the actively transcribing polymerase II (RNAPII) [18]. The appearance of RNA sequencing and Global Run-On sequencing (GRO-seq) technologies revealed the existence of new types of RNA species including enhancer RNAs (eRNAs) [19,20]. These short RNAs are transcribed from active enhancers only and follow the induction profile of their target genes. Recently, it has been shown that the modified version of GRO-seq by enriching 5′-capped RNAs is a very powerful tool to identify active transcriptional regulatory elements based on its improved eRNA detection ability. The analysis of such datasets demonstrated the enrichment of expression quantitative trait loci, disease-associated polymorphisms, H3K27ac and transcription factor binding sites at transcriptionally active regulatory elements [21]. According to these findings, eRNAs represent an accepted functional enhancer feature [22]. Hence, it has already been exploited to assign the active enhancer element repertoire of certain transcription factors, such as ESR1 [23] or RXR [24] to their target genes genome-wide.

Increasing evidence suggests that genes and their enhancers are located in functional domains separated from each other by proteins bound to insulator sequences. Insulators provide a barrier function to prevent repressive heterochromatin from spreading into a neighboring domain, provide an enhancer-blocking function when positioned between the enhancer and promoter, and allow three-dimensional looping of genomic regions [25,26]. CCCTC-binding factor (CTCF) is known to act as an insulator, but also implicated in the regulation of higher order chromatin structure. Recent studies investigating the spatial organization of the genome show that the direction of the CTCF motif determines the 3D folding of the genome [27]. In addition, it has been also shown that CTCF anchors play a vital role in connecting enhancers to their target gene promoter [28].

Based on advanced sequencing technologies, in 2014 the ENCODE (Encyclopedia of DNA elements) Consortium has released genome-wide datasets of the mouse genome [29]. These studies aimed to identify the *cis*-acting element repertoire of several human and mouse cell lines. In addition, they provided genome-wide data also from various mouse tissues.

In the present work we decided to identify those enhancers which regulate the expression of *Tgm2* in dying thymocytes exposed to retinoids, TGF-β and/or following activation of the adenylate cyclase signaling pathway. Having access to the ENCODE generated datasets from mouse thymus, first we integrated DNase-seq and ChIP-seq results against CTCF, H3K4me3, H3K4me1 and H3K27ac in order to map the putative regulatory elements participating in the regulation of *Tgm2*. Then by measuring their basal and induced eRNA levels, we determined which of them are functional. Finally, we analyzed the basal and induced binding of SMAD4, retinoic acid (RAR), retinoid X (RXR) receptor and cAMP response element binding protein (CREB), transcription factors mediating the effects of the retinoid, TGF-β and adenylate cyclase signaling pathways, to these enhancers. Our study describes the thymocyte-specific enhancer elements of *Tgm2* and presents a novel strategy to identify and characterize the functional contribution of individual enhancers to gene regulation.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except indicated otherwise.

2.2. Experimental animals

Most of the experiments were carried out with 4-wk-old C57Bl/6 mice. Mice were maintained in specific pathogen-free condition in the Central Animal Facility of our university, and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB).

2.3. Thymocyte cultures

Isolated thymocytes (1×10^7/ml) were cultured in RPMI medium 1640 supplemented with 10% charcoal stripped FBS, 2 mM glutamine, 1 mM Na pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in 5% CO_2. Cells were exposed to 0.3 μM 9-cis retinoic acid (9cRA) (Tocris Bioscience, Eching, Germany), 1 μM AM580, an RAR agonist, 100 nM LG268, an RXR agonist analogue alone, or in combination with 100 μM db-cAMP, a cell permeable cAMP or with 5 ng/ml recombinant human TGF-β1 (AbD Serotec, Kidlington, UK) and for the indicated time periods.

2.4. Real-time quantitative PCR (qRT-PCR) for detecting eRNA levels

For this purpose we designed eRNA specific primer pairs targeting the enhancer sequences upstream to their DNase hypersensitive sites, but on the enriched histone modifications (Supplementary Fig. 1). We used primer3 software to design the eRNA specific primers with standard conditions except the followings: amplicon length: 90–150 bp; Tm: 59–60 °C [30]. RNA was isolated with Trizol Reagent according to the manufacturer's guidelines. For eRNA measurements, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Budapest, Hungary) was used and transcript quantification was performed by Quantitative Real-Time PCR reaction using SYBR green dye (gb SG PCR Master Mix, Generi Biotech, Czech Republic). RNA was isolated with the same Trizol based method, but samples were digested with DNaseI enzyme for 1 h at 37 °C. RNA was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Budapest, Hungary) and quantification was performed with LightCycler® 480 (Roche, USA). The following PCR program was used for enhancer RNA detection: 95 °C-3 min; 95 °C-30 s; 60 °C-