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The retrotransposon gag domain containing protein Rgag4 is an Ikaros target in the pituitary

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ABSTRACT

Previous studies have established the common and critical involvement of the zinc finger protein Ikaros in lymphoid and pituitary cell development and expansion. Key to the assembly of several transcriptional networks, we have demonstrated up-regulation of Ikaros and its interacting partner the C-terminal Binding Protein (CtBP) in response to hypoxia. This prompted us to explore common transcriptional targets using a chromatin immunoprecipitate (ChIP) screen of DNA from pituitary corticotroph cells. This strategy yielded a finite list of targets common to both transcription factors that included the metalloprotease ADAMTS10. In this report, we focus on validation of a second candidate target, the retrotransposon gag domain containing protein Rgag4. We identified the ability of Ikaros to bind the Rgag4 promoter, influence its transcriptional activity, and induce endogenous gene expression. Robust expression of Rgag4 was noted in the anterior lobe of the pituitary gland which was diminished in Ikaros knockout mice. Down-regulation of Rgag4 resulted in profound reduction of hormone gene expression with diminished ACTH secretion, recapitulating the effect of Ikaros deficiency in knockout mice. The results introduce Rgag4 to the repertoire of effectors serving to couple the chromatin remodeler Ikaros with the hormonal stress response.

1. Introduction

We have previously shown that the zinc-finger DNA-binding protein Ikaros (Ik), recognized for its role in lymphoid system development (Cortes et al., 1999; Wang et al., 1996; Winandy et al., 1999), is also expressed in the anterior pituitary gland where it directs cell lineage expansion (Ezzat et al., 2006, 2005a, 2005b). Consistent with this prediction, Ikaros-deficient mice show reduced growth and succumb to increased mortality due to deficient development of the hypothalamic-pituitary-adrenal axis (Ezzat et al., 2005b). Importantly, reconstitution with wild-type bone marrow rescues the immunodeficiency of these mice but does not correct the endocrine dysfunctions (Ezzat et al., 2005b). Indeed, administration of pituitary-regulated hormones, including glucocorticoids, is required to normalize survival, and growth hormone is required to restore somatic growth of Ikaros-deficient mice (Ezzat et al., 2005b, 2006). These findings provided the framework for a model that implicates the chromatin remodeler Ikaros at the center of immune and endocrine system development and interactions (Chrousos and Kino, 2005).

Ikaros interacts with several components of NURD complexes including the C-terminal Binding Protein (CtBP), as was initially described in thymic extracts (Koipally and Georgopoulos, 2000) and subsequently in pituitary cells (Dorman et al., 2012). Of note, the interaction between CtBP and Ikaros is induced in response to hypoxia (Dorman et al., 2012) while loss of CtBP impairs the Ikaros response. This structural and functional interaction prompted us to seek common transcriptional targets for Ikaros and CtBP in pituitary cells using chromatin immunoprecipitation (ChIP) assays for promoter array hybridization (Shen et al., 2017). Using a ChIP-on-chip approach, anti-Ikaros and anti-CtBP ChIPs from the same cells were amplified for LDL-R recovery (Dorman et al., 2012; Loeper et al., 2008) and for promoter array hybridization (Shen et al., 2017). As reported previously, this approach led to a finite list of candidate promoters which included the metalloprotease ADAMTS10 (Shen et al., 2017). Recognized for its ability to control growth factor signaling, ADAMTS10 was shown to influence

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enzymatic function to control pituitary hormonal activity (Shen et al., 2017). In this report, we pursue a second common transcriptional target that emerged from the initial Ik/CtBP ChIP screen of AtT20 cells, the Retrotransposon gag domain containing 4 (Rgag4) which hitherto has not been examined in the pituitary.

2. Materials and methods

2.1. Cells and cell counting

Mouse pituitary corticotroph AtT20 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Burlington, Canada) and antibiotics as previously described (Shen et al., 2017). Cells were plated at a density of 1.0×10^4 in 6 multi-well plates for detection by alamar blue for cell counting at the indicated time points.

2.2. Plasmids and transfections

Expression vectors encoding Ikaros 1 (Ik1) or its non-DNA binding isoform Ikaros 6 (Ik6) in pcDNA3.1 were used as previously described (Ezzat et al., 2005a). To down-regulate Rgag4, cells were transfected with scrambled sequence or Rgag4 shRNA plasmids (GenePharma Co., Ltd., Shanghai, China) using GeneJuice Transfection Reagent (Merck KGaA, Darmstadt, Germany). Rgag4 knockdown clones were selected with Hygromycin B (Wisent Bio Products Inc, St-Bruno, QC) and their expression levels compared by Western blotting.

2.3. ChIP (chromatin immunoprecipitation)

ChIP assays were performed as directed by the manufacturer's recommendations (Millipore, Temecula, CA). In short, for cross-linking histones to DNA, formaldehyde was added directly to culture media, cells were washed, scraped, and resuspended in lysis buffer for sonication to shear DNA fragments. This was followed by centrifugation, then the cell supernatant was diluted and a portion of the supernatant was used for DNA quantification and input DNA detection by PCR. The remaining cell supernatant was cleared with salmon sperm DNA/protein A-agarose beads. Immunoprecipitation was performed by incubation with either anti-Ikaros antibody (Santa Cruz Biotechnology) or anti-CtBP1 antibody (Sigma, Saint Louis, MO) overnight at 4 °C with constant rotation. Negative controls were obtained by incubation with either normal rabbit or goat IgG. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. ChIP DNA and input DNA were amplified using the GenomePlex complete whole genome amplification kit (Sigma, Saint Louis, MO) by PCR using LDL-R primers as previously described (Shen et al., 2017). Primer sequences for Rgag4 amplification are shown at the top of Fig. 1.

2.4. Promoter array and analysis

Promoter array analysis was performed with the 385K NimbleGen Microarray Services (Roche, Reykjavik, Iceland), database for Annotation, Visualization and Integrated Discovery (DAVID) as reported earlier (Shen et al., 2017). Putative binding sites of transcription factors Ikaros or CtBP1 in the mouse Rgag4 (NM_183318) gene promoter sequences were submitted to transcription factor databases (www.cbrc.jp/research/db/TFSEARCH.html and www.cbiliupenn.edu/cgi-bin/tess/tess?RQ=SE A-FR-Query). We followed the convention of using zero as the first base of the coding sequence in exon 1or exon 2 for sequence coordinates.

2.5. Promoter luciferase reporter assays

The 1682 bp 5' region of the mouse Rgag4 promoter was amplified from AtT20 cells (Qiagen, Hilden, Germany) for cloning into the PcR2.1 TA vectors (Invitrogen, Carlsbad, CA). Orientation and sequence was confirmed by restriction digestion and nucleotide sequencing. Promoter fragments were inserted into the *Kpn*I and *Bgl*II sites of pGL3-enhancer-luciferase vector (Promega, Madison, WI). Reporter plasmids were confirmed by restriction digestion and purified by column chromatography (Qiagen, Gaithersburg, MD) for transfection.

2.6. Western blotting

Protein was extracted and quantified by the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Inc., Ontario, Canada). Samples were resolved on 10–12% polyacrylamide gel and electrophoretically transferred onto polyvinylidene fluoride membranes for hybridization and chemiluminescent detection as previously described (Shen et al., 2017). Protein levels were normalized to β-actin (1:1000; Cell Signaling). The antibodies used included: rabbit polyclonal anti-Rgag4 antibody (Novus Biologicals, NBP1-93561) 1/1500, rabbit polyclonal against ACTH (Abcam Ab74976) 1/10,000; rabbit polyclonal against Ikaros (Abcam ab 26083) 1/1000; goat polyclonal against Ikaros (Santa Cruz 612044) 1/1500; rabbit polyclonal against CtBP1 C-terminal (Sigma C8741) 1/2000.

2.7. RNA expression analysis

RNA was isolated using RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Ontario). The integrity of RNA was established by the amplification of GAPDH transcripts (Applied Biosystems, Life Technologies Corp., California, USA). Primer sequences were as follows: Rgag4 forward GAAGCGGCGGGAAATCTCAA, reverse GAAGGGCATTTAACTCCTCTCG; GAPDH forward GAAGGGCTCATGACCACAGT, reverse GGATGCAGGGATGATGTTCT. Thermal cycling conditions were: 25 °C for 10 min, 37 °C for 2 h, 85 °C for 5 min. Real-time PCR reactions used RT^{2}SYBR green with ROX master Mix (SABiosciences, Frederick). Primers yielded a single product, and all dissociation curves with a single peak were confirmed with real-time PCR products run on 1% agarose gels. The results were analyzed using a comparative method, normalized to endogenous reference following the formula $2^{-\Delta\Delta Ct}$, where Ct represents the threshold cycle, indicating the fractional cycle number at which the amount of amplified target reaches fixed threshold.

2.8. Mouse histology and immunohistochemistry

Primary formalin-fixed, paraffin-embedded normal mouse pituitary glands were used for immunohistochemical stains as described previously (Shen et al., 2017). The rabbit polyclonal anti-Rgag4 antibody (Novus Biologicals, NBP1-93561) was incubated overnight at 1:150 dilution after citrate pretreatment, avidin/biotin block and casein block and detected using the Immpress Rabbit detection kit (Vector Laboratories, Burlingame CA). Omission of primary antibody was used to exclude non-specific reactivity.

2.9. Statistical analysis

Data are shown as mean ± standard deviation. Differences were assessed using the Student's paired *t*-test at the same time point. Significance was based on a value of $p \leq 0.05$.

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