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The adaptor protein ARA55 and the nuclear kinase HIPK1 assist c-Myb in recruiting p300 to chromatin

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ABSTRACT

LIM-domain proteins, containing multiple cysteine-rich zinc finger-like motifs, have been shown to play diverse roles in several cellular processes. A common theme is that they mediate important protein-protein interactions that are key to their function. Androgen receptor-associated protein 55 (ARA55) belongs to this family of bridging proteins containing four C-terminal LIM domains. It has a dual role with functions both at focal adhesions and in the nucleus, apparently shuttling between the two compartments. In the present work, we have expanded our understanding of its nuclear functions by showing that it interacts with three nuclear regulators not previously linked to ARA55. We first identified ARA55 as a novel interaction partner of the nuclear kinase HIPK1 and found that ARA55, like HIPK1, also interacts with the transcription factor c-Myb. In search of a function for these associations, we observed that the coactivator p300 not only binds to c-Myb, but to ARA55 as well. When combined, c-Myb, p300, HIPK1 and ARA55 caused strong synergistic activation of a chromatinized reporter gene. In parallel, all partners, including p300, were efficiently recruited to chromatin at the c-Myb-bound promoter. Consistent with this cooperation, we found that c-Myb and ARA55 share a common set of target genes in an osteosarcoma cellular context. We propose that ARA55 and HIPK1 assist c-Myb in recruiting the coactivator and acetyltransferase p300 to chromatin.

1. Introduction

TGFB1I1/ARA55/HIC-5 is a LIM-domain protein and a member of the paxillin family, implicated in several different processes in the cell, such as cell growth, proliferation, migration, differentiation and senescence, with a defined role in focal adhesion, Wnt and TGFβ signalling, and nuclear receptor activation [1–3]. This variety of functions is reflected in several designations of the gene, TGFB1I1 (Transforming Growth Factor Beta 1 Induced Transcript 1), ARA55 (Androgen Receptor-Associated Protein of 55 kDa; preferred designation in this work), or HIC-5 (Hydrogen Peroxide-Inducible Clone 5 Protein). The many functions are explained by the protein operating as a molecular adapter, coordinating multiple protein-protein interactions. An important feature of the gene is its response to TGFβ and oxidative stress. Since the protein has functions both at the cell membrane and in the nucleus, it may convey signals between these compartments [4].

While the cytoplasmic role of ARA55 relates to it functioning as a molecular scaffold at focal adhesions [1,2,5–7], its nuclear functions are linked to ARA55 operating as a coactivator, in particular towards nuclear receptors regulating glucocorticoid, androgen, and progesterone-responsive gene programs [3,8–12]. Inactivation of ARA55 resulted in reduced androgen receptor (AR) activity in prostate cancer cell lines [3,9,13]. Conversely, aging was found to up-regulate ARA55 in stromal cells, inducing androgen-mediated prostate cancer cell proliferation and migration [14]. The adaptor protein shuttles between the cytosolic (focal adhesion complexes) and the nuclear compartments, with TGFβ and oxidative stress promoting its nuclear localization by inhibiting its nuclear export [2]. Once in the nucleus, ARA55 functions as a

Abbreviations: 2KR, double mutant of c-Myb (K503R + K527R); AR, androgen receptor; ARA55, AR coactivator with a molecular mass of 55 kDa; c-Myb, v-myb avian myeloblastosis viral oncogene homolog; ChIP, chromatin immunoprecipitation; CoIP, co-immunoprecipitation; DBD, DNA-binding domain; GST, glutathione S-transferase; HAT, histone acetyltransferase; hcM, human c-Myb; HIPK1, homeodomain interacting protein kinase 1; KIX, the CREB and c-Myb interaction domain of p300; LIM, a cysteine-rich zinc finger-like motifs named after its initial discovery in the proteins **L**in11, **I**sl-1 & **M**ec-3; p300, E1A binding protein p300 or histone acetyltransferase p300; PAGE, polyacrylamide gel electrophoresis; SIM, SUMO-interaction motifs; SUMO, small ubiquitin-related modifier; TAD, transactivation domain; TF, transcription factor

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transcriptional co-activator for AR and other specific transcription factors (TFs), such as nuclear receptors, SMADs and Sp1 [3,12,15].

ARA55 is linked to cancer development in several ways. ARA55 expression becomes induced by TGFβ, contributing to epithelial mesenchymal transition (EMT), cell migration, and invasion [16,17]. Furthermore, ectopic expression of ARA55 is sufficient to promote normal human breast epithelial cells to undergo EMT [16]. Both Paxillin and ARA55 impact the canonical metastasis cascade at several levels through their ability to coordinate plasticity and efficient mesenchymal and amoeboid invasion, respectively [18]. Interestingly, the protein seems to have distinct effects in different tissues and operates both as an oncogene and a tumour suppressor, dependent on its cellular context. Whereas ARA55 in breast cancer promotes invasion and cancer development [16,19], its expression is reduced in colon tumours. In gut epithelial cells, it collaborates with PPARγ to induce expression of markers of gut maturation, consistent with a role as a tumour suppressor [10].

In the present work, we have expanded the list of nuclear partners of ARA55 to include the kinase HIPK1 and the TF c-Myb. Homeodomain-interacting protein kinases (HIPK1-3) constitute a small family of nuclear serine/threonine kinases, initially identified as corepressors for homeodomain TFs of the NK class [20]. HIPK2 is the best-studied member of the family [21]. Judged from its expanding number of phosphorylation targets [22–24], it seems that the HIPK family plays critical roles in transcriptional regulation. It has also been linked to apoptosis, DNA-damage [22] and recently to cytokinesis [25].

The other nuclear partners of ARA55 analysed in this work is c-Myb, belonging to a group of early hematopoietic TFs. It operates as a regulator of stem and progenitor cells in the bone marrow as well as in colonic crypts, and in a neurogenic region of the adult brain [26,27]. It is required for normal adult hematopoiesis and plays a direct role in lineage commitment, cell cycle progression, and differentiation of both myeloid and B and T lymphoid progenitor cells [28,29]. Moreover, c-Myb controls intestinal stem cell genes and self-renewal [30] and is a key regulator that permits early multilineage differentiation of airway epithelial cells [31]. In an oncogenic context, c-Myb enhances the proliferation and blocks differentiation of the cancer cells. Clinical studies have revealed strong links between c-Myb aberrations and human cancer, such as acute myelogenous leukemias, melanomas, and breast, colon and pancreatic carcinomas [26,27,32,33]. Zuber et al. identified c-Myb as a critical mediator of oncogene addiction in acute myeloid leukemia (AML) [34]. Furthermore, c-Myb was found to cause enhanced motility and invasion and to be linked to the EMT response [35–37].

Key coregulators of c-Myb and many other TFs are the acetyltransferases p300 and CBP, acting both as bridges to the basal transcriptional apparatus and facilitating transcription by acetylation of chromatin and its interacting partners [38–42]. Abolishing the interaction between c-Myb and p300/CBP results in non-functional hematopoiesis with thrombocytosis and impaired lymphoid development [43]. A mutation preventing interaction between c-Myb and p300 also prevents transformation and leukemia induction by human AML oncogenes [44]. c-Myb is also reported to bind to the methyl transferase MLL through its complex partner menin, thereby promoting MLL-associated leukemogenesis [45].

In the present work, we first identified ARA55 as a novel interaction partner of the nuclear kinase HIPK1. Together they had a coactivator effect on c-Myb. Interaction assays showed that ARA55 physically associates with c-Myb as does HIPK1. In search of a function for these associations, we detected a novel physical interaction between p300 and ARA55. Together, c-Myb, p300, HIPK1 and ARA55 caused strong synergistic activation of a chromatinized reporter gene, to which they were efficiently recruited. Our data suggest that ARA55 and HIPK1 assist c-Myb in recruiting p300 to chromatin. In support of this hypothesis, we found that c-Myb and ARA55 share a common set of target genes in a cell type where both are expressed.

2. Materials and methods

2.1. Cell culture, transfection and luciferase assays

Five cell lines were used: CV-1 (ATCC ® CCL-70™ *Cercopithecus aethiops* kidney Normal), COS-1 (ATCC ® CRL-1650™ *Cercopithecus aethiops* kidney), K562 (ATCC® CCL-243™ *Homo sapiens* bone marrow, chronic myelogenous leukemia), HEK-293 (ATCC ® CRL-1573™ *Homo sapiens* embryonic kidney) and U2OS (*Homo sapiens*, epithelial). The latter was a kind gift from Dr. Ian G. Mills, Centre for Molecular Medicine Norway. The cells were grown and transiently transfected with the indicated plasmids as previously described [46,47]. Reporter assays in transiently transfected CV1 and HEK293 cells, the latter stably transfected with a 5× Gal4-luciferase reporter, were performed in triplicate (24-well trays, 2×10^4 CV-1 cells/well or 3.2×10^4 HEK293-c1 cells/well) using Luciferase Assay Reagent (Promega), each triplicate repeated in three independent experiments.

K562 cells stably expressing V5-tagged ARA55 were generated by standard methods. K562 cells were transfected with linearized pCIneo-ARA55-V5 using the Amaxa kit V (Amaxa, Cologne, Germany) according to manufacturer's guidelines. K562 cells stably expressing tagged ARA55-V5 were selected by G418 (400 μg/ml) for 2 weeks before selecting single clones for one week. Stable expression was confirmed by Western blotting.

2.2. Plasmid constructs

The construct for mammalian expression of V5-tagged human ARA55, pCIneo-ARA55-V5, was made by subcloning a PCR-amplified ARA55 cDNA, in which a V5-encoding sequence was added through the reverse PCR primer. The pGEX-6P2-ARA55-V5 was constructed with a similar approach and used to produce recombinant GST-ARA55-V5. Truncated versions (GST-ARA55 (1 − 213) and GST-ARA55 (214–461)-V5) were expressed from the plasmids pGEX-6P2-ARA55-N and pGEX-6P2-ARA55-V5-C, which were similarly constructed from PCR amplified ARA55 cDNA.

Plasmids for expression of HIPK1 full-length, wild-type and kinase-dead mutant K219A, has been described [48]. To make the Gal4-fusion bait plasmid for the yeast two-hybrid screening, we subcloned a region of HIPK1 encoding the C-terminal half of the protein (amino acids 632–1209) into pDBT [49] to create pDBT-hHIPK1-BC. Plasmids for expression of p300 full-length and domains have been described [50]. The plasmids encoding Gal4-fusions and GST-fusions of human c-Myb have been described [46,50,51]. The same apply to the reporter plasmids used, pGL4b-3xMRE(GG)-myc [46] and pGL3-GATA2 [52].

2.3. Yeast two-hybrid screening

The two-hybrid screening was performed as previously described [48,53–55]. The interactions were confirmed by transformation of pDBT-hHIPK1-BC and rescued interaction candidates in pACT2 into yeast strains of opposite mating type followed by mating. Diploids were grown on yeast minimal medium supplemented with 0–5 mM 3-aminotriazole and lacking the selection amino acids or adenine.

2.4. Antibodies

For immunoblot detection the following antibodies were used: mouse anti-V5 monoclonal antibody (R96025, Invitrogen), mouse anti-FLAG M2 monoclonal antibody (F3165, Sigma-Aldrich), mouse anti-Myb (5E11, [56]), rabbit anti c-Myb H141 (sc7874, Santa Cruz Biotechnology), mouse anti GAPDH (AM4300, Invitrogen), anti-mouse IgG-HRP (715-035-150, Jackson ImmunoResearch), and anti-rabbit IgG-HRP (711-035-152, Jackson Immuno-Research). Protein A Dynabeads (10002D, Invitrogen) and rabbit anti-p300 (C-20, SC-585 X, Santa Cruz Biotechnology) were used for immunoprecipitation. In the