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A conserved patch of hydrophobic amino acids modulates Myb activity by mediating protein–protein interactions

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ABSTRACT

The transcription factor c-Myb plays a key role in the control of proliferation and differentiation in hematopoietic progenitor cells and has been implicated in the development of leukemia and certain non-hematopoietic tumors. c-Myb activity is highly dependent on the interaction with the coactivator p300 which is mediated by the transactivation domain of c-Myb and the KIX domain of p300. We have previously observed that conservative valine-to-isoleucine amino acid substitutions in a conserved stretch of hydrophobic amino acids have a profound effect on Myb activity. Here, we have explored the function of the hydrophobic region as a mediator of protein–protein interactions. We show that the hydrophobic region facilitates Myb self-interaction and binding of the histone acetyl transferase Tip60, a previously identified Myb interacting protein. We show that these interactions are affected by the valine-to-isoleucine amino acid substitutions and suppress Myb activity by interfering with the interaction of Myb and the KIX domain of p300. Taken together, our work identifies the hydrophobic region in the Myb transactivation domain as a binding site for homo- and heteromeric protein interactions and leads to a picture of the c-Myb transactivation domain as a composite protein binding region that facilitates interdependent protein–protein interactions of Myb with regulatory proteins.

1. Introduction

Vertebrate Myb proteins constitute a small family of highly conserved transcription factors (c-Myb, A-Myb and B-Myb) that play key roles in the control of proliferation and differentiation of various cell types [1,2]. The founding member of the Myb family, v-Myb, is a truncated and mutated version of c-Myb and was originally identified as a retroviral protein responsible for the transforming potential of the avian myeloblastosis virus (AMV) [3]. A large body of work has since shown that c-Myb acts as a critical transcription factor in the hematopoietic system. c-Myb is highly expressed in immature, proliferating hematopoietic cells and is down-regulated during terminal differentiation [1,2]. Constitutive expression of c-Myb blocks hematopoietic differentiation while disruption of the gene in mice leads to defects of multiple hematopoietic lineages and embryonic death [4]. Numerous studies have shown that c-Myb regulates the expression of specific target genes, thereby affecting proliferation, differentiation and apoptosis [2].

Although Myb was discovered in the context of a chicken leukemia virus recent work has provided evidence that c-Myb is also involved in the development of leukemia and certain non-hematopoietic tumors in humans. Rearrangements of the c-*myb* locus have repeatedly been observed in acute lymphoblastic leukemia (T-ALL) [5,6]. Translocations between the *MYB* and *NFIB* genes occur in a large percentage of adenoid cystic carcinomas, leading to the expression of MYB-NFIB fusion proteins [7]. Deciphering the molecular function(s) of Myb in transcriptional regulation is therefore a prerequisite for understanding how deregulation of c-Myb contributes to tumor development.

Transcriptional activation of target genes by Myb has been studied in detail [8–16]. Myb activity is highly dependent on the interaction with the coactivator p300. Binding of p300 to Myb is mediated by a LXXLL amino acid motif that is located in the transactivation domain of Myb and interacts with a hydrophobic cleft on the surface of the KIX domain of p300 [17]. Mutation of the LXXLL motif disrupts the interaction of Myb and p300 and strongly reduces Myb activity and causes defects of the hematopoietic system [18–21]. The Myb-p300 interaction has recently also been shown to be a potential target for small-molecule inhibitors of Myb activity [22,23]. Close to the N-terminal side of the p300 binding region lies a conserved patch of hydrophobic amino acids that plays a role in chromatin remodeling by Myb (Fig. 1). We have previously analyzed the functional consequences of amino acid substitutions in the hydrophobic region that differ between the highly oncogenic v-Myb and c-Myb and are thought to contribute to the oncogenic potential of v-Myb. These studies showed that the substitution of two valine residues by isoleucine in the hydrophobic region of v-Myb disrupts the ability of v-Myb to stimulate the activity of an enhancer driving the expression of the Myb target gene *mim-1* and to remodel its nucleosomal organization [14]. We therefore speculated that the hydrophobic

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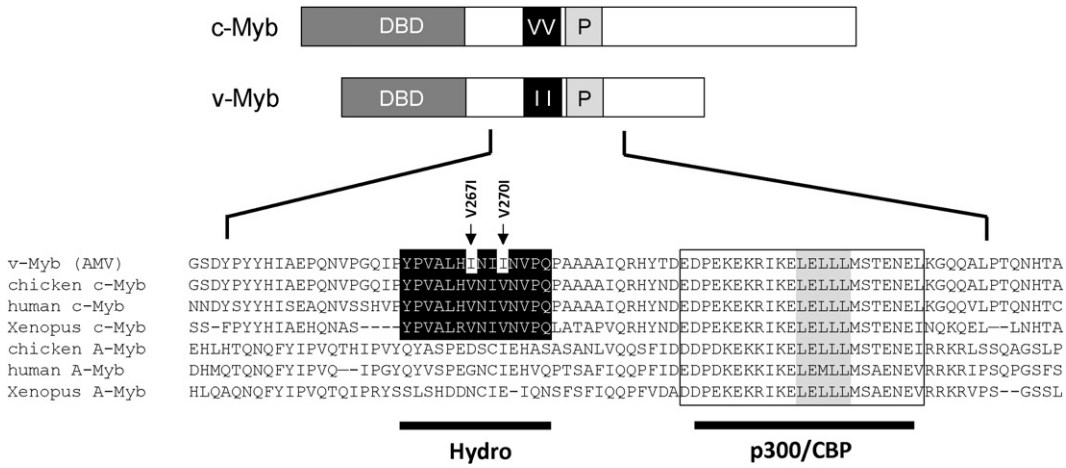

Fig. 1. Amino acid sequence of the Myb transactivation domain. The domain structure of c-Myb and v-Myb is shown schematically at the top. The hydrophobic region is shown in black. DBD and P mark the DNA-binding domain and the p300-binding site. The amino acid sequence of the hydrophobic region and the p300-binding site of c-Myb and A-Myb proteins from different species is shown below. The valine-to-isoleucine substitutions in the hydrophobic region and the LXXLL motif for p300-binding are highlighted.

region might serve as a protein–protein interaction surface that acts together with the p300-binding site in the Myb transactivation domain. Independently of our work, the hydrophobic region was recently identified as a SUMO-interacting motif (SIM) suggesting that it might be involved in the non-covalent binding of sumoylated proteins to Myb [24].

We have now studied the ability of this hydrophobic region to support protein–protein interactions in more detail. We show that this region mediates homo- and heteromeric interactions with Myb itself and with the histone acetyl transferase Tip60. Furthermore, we demonstrate that these interactions are sensitive to the valine-to-isoleucine substitutions and negatively affect the activity of Myb.

2. Material and methods

2.1. Transfections

Transfections of the QT6 quail fibroblast and HD11 chicken macrophage cell lines were performed by calcium-phosphate co-precipitation and reporter assays were performed 16 to 24 h after transfection as described [10]. The Myb responsive luciferase reporter plasmid pGL4-5xMRE(GG)-Myc was obtained from O. Gabrielsen [25]. The β-galactosidase reporter plasmid pCMVβ (Clontech) was used as control for the transfection efficiency. Luciferase values were always normalized against the β-galactosidase activity to correct for differences in transfection efficiency. All reporter studies were performed in at least 3 independent experiments, with replicate transfections in each experiment. The expression of the endogenous *mim-1* and ribosomal protein S17 mRNAs was analyzed by northern blotting as described before [10].

2.2. Expression vectors

Expression vectors pcDNA3-v-MybRev and pcDNA3-v-MybEP have been described [14]. The proteins encoded by these vectors differ only in the amino acid sequence of the hydrophobic region and are here referred to as v-Myb(VV) and v-Myb(II), respectively. pEYFP-C1-H(VV) and pEYFP-C1-H(II) were generated by cloning an EcoRI/PvuII fragment from pcDNA3-v-MybRev and pcDNA3-v-MybEP into pEYFP-C1, thereby fusing EYFP coding sequences to v-Myb sequences between the end of the DNA-binding domain and the end of the hydrophobic domain (amino acids 116–207). The proteins encoded by these plasmids are referred to as YFP-H(VV) and YFP-H(II), respectively. YFP-H(ANAA) was generated by point-directed mutagenesis. pEYFP-C1-Myb-short was generated by cloning a PCR fragment containing coding sequences for Myb amino acids 116–188 into pEYFP-C1. The protein encoded by this construct is referred to as YFP-short. pcDNA3-6xMyc-vMyb(N) and pcDNA3-6xMyc-vMybN + H(II) encode v-Myb amino acids 1 to 135 and 1 to 207, respectively, fused to an N-terminal 6-fold Myc-tag. The proteins encoded by these plasmids are referred to as v-MybN and v-MybN + H(II), respectively. pCDNA3-6xMyc-vMyb(C) encodes v-Myb amino acids 205 to 382 fused to a 6-fold Myc-tag, which is referred to as v-MybC. pKIX/VP16 encodes a fusion protein of the VP16 transactivation domain and the KIX domain of p300 (amino acids 556–652). The expression vector for human TIP60 pCMV2B-Flag-hTIP60 was obtained from A. Gewirtz [26]. Bacterial expression vector for GST-H(II) was prepared by cloning to v-Myb sequences between the end of the DNA-binding domain and the end of the hydrophobic domain (amino acids 116–207) into pGex-6P2. The expression vector for the bacterial GFP-TAD protein (containing v-Myb amino acids 115–320) has been described [22].

2.3. GFP-trap experiments

QT6 fibroblasts were transfected with the desired expression vectors encoding YFP or YFP-tagged proteins. The cells were lysed 24 h post transfection in egg lysis buffer (ELB) (120 mM sodium chloride, 50 mM Tris/HCL, pH 7.5, 20 mM sodium fluoride, 1 mM EDTA, 6 mM EGTA, 15 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Nonidet P-40) and centrifuged for 20 min at 14,000 ×*g*. An aliquot of the supernatant was retained as control for the total cell extract. The remaining supernatant was incubated with the GFP-trap beads (Chromotec, München) for 3 h at 4 °C. Beads were washed three times using ELB buffer, boiled in sodium dodecyl sulfate (SDS) sample buffer and analyzed together with the input samples by SDS polyacrylamide gel electrophoresis and western blotting, using appropriate antibodies.

2.4. Far western blotting

Far western blotting was performed as described before [27]. Briefly, bacterially expressed GST and GST-H(II) was purified as described using glutathione sepharose, fractionated by 10% SDS-PAGE and blotted onto nitrocellulose. The blots were first incubated for 2 h with incubation buffer (10 mM Tris–HCl, pH 7.4; 1 mM EDTA; 150 mM NaCl; 0.2% NP-40) containing 5% skim milk powder. Subsequently, blots were incubated for 2 h with incubation buffer containing 1% skim milk powder and a purified bacterial GFP-Myb fusion protein. Unbound protein was removed by three washes for 10 min in incubation buffer, followed by staining as a regular western blot using anti GFP antibodies. All steps were carried out at room temperature.

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