FEBS Letters 582 (2008) 379–384

Alternative mRNA splicing of SAP30L regulates its transcriptional repression activity

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Received 4 October 2007; revised 26 November 2007; accepted 29 November 2007

Available online 10 December 2007

Edited by Ivan Sadowski

Abstract **Covalent modification of histones regulates chromatin structure and gene expression. Sin3A mediates the association of histone deacetylase enzymes with a large number of sequence-specific transcriptional repressors. In this study we characterized three novel transcripts of SAP30L, a recently identified Sin3A-associated protein. These splice variants show significant differences in transcriptional repression capabilities and associating histone deacetylase activities. Furthermore, they differ in binding to Sin3A and in subcellular localization when transiently transfected. These data suggest that the transcriptional repression of a Sin3A corepressor complex can be regulated not only by sequence-specific transcriptional repressors, but also by modification of associated proteins, such as SAP30L.**

Keywords: Histone; Repression; SAP30L; Splicing; Transcription

1. Introduction

In eukaryotic cells, genetic information is organized in a highly conserved structural polymer, chromatin. The primary unit in chromatin is the nucleosome, composed of 146 bp of DNA wrapped around an octamer of histone proteins. Compacted chromatin allows efficient storage of genetic information but simultaneously forms a sterically hindered environment for the assembly of the transcription machinery. Packaging and unpackaging of chromatin is thus intricately regulated. One major mechanism altering the chromatin structure and gene expression is covalent modification of histone tails by acetylation, methylation, phosphorylation, sumoylation, ubiquitination, glycosylation, or ADP-ribosylation [1–3]. Histone deacetylation is a well-conserved mechanism for silencing gene expression [4]. Histones are typically deacetylated by multiprotein complexes containing histone deacetylases (HDACs) and corepressor proteins which vary depending on the specific complex. Corepressor proteins are instrumental in establishing the specificity of a given complex. This is nicely demonstrated by retinoblastoma protein, which recruits HDAC complex to E2F transcription factors, leading to repression of the transcription of specific E2F-targeted genes [5].

Sin3A is a large protein composed of protein–protein interaction domains and it mediates the association of heterogeneous HDAC complexes and other chromatin-modifying proteins with transcriptional repressors [6]. HDAC1 and HDAC2, the histone-binding proteins RbAp46 and RbAp48, Sin3A-associated proteins SAP18, SAP30, SAP130, SAP180, and SDS3 are widely identified as core components in the Sin3A-HDAC corepressor complex. Of the Sin3A-binding proteins, SAP30 (Sin3A-associated protein 30) and SAP30L (L for like) share 70% identity at sequence level. SAP30 is a member of the Sin3A core complex and is thought to bridge and stabilize molecules in the complex [7,8]. SAP30L was identified as a mRNA transcript with increased expression due to differentiation of T84 colon carcinoma cells by transforming growth factor β (TGF-β) [9]. Our group has recently reported that SAP30L associates with the core members of the Sin3A corepressor complex and is able to induce transcriptional repression presumably through Sin3A and class I HDACs [10]. In this study, we investigated the mRNA splice forms of SAP30L and report that the transcriptional repression mediated by SAP30L can be critically influenced by alternative mRNA splicing.

2. Materials and methods

2.1. Cloning and constructs

Human SAP30L cDNA has been described elsewhere [9]. Splice variant cDNAs were obtained from the following IMAGE clones: 5254-i19 (ΔEx2), 4554-h16 (ΔEx2Δ5aa), and 6022-i24 (ΔEx3,4). They were subcloned into a pcDNA3.1-myc-his expression vector (Invitrogen) for mammalian transfections, and pCMV-BD (Stratagene) and pGEX-4T1 (Amersham Bioscience) vectors for the production of Gal4- and GST-fusion proteins, respectively. The 5-residue deletion mutant (Δ109–113) was created using the QuickChange® Site Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Accuprime® Pfx (Invitrogen) high-fidelity DNA polymerase was used in PCR amplification and all of the constructs were confirmed by sequencing. pCS2 + MT-mSin3A plasmid and luciferase reporter vector with five Gal4-binding sites and under the control of 14D promoter (5xGal4-14D-LUC) [8] were generous gifts from D. Ayer (Salt Lake City, USA).

2.2. Identification of mRNA splice forms

RNA isolated (RNeasy Mini Kit, Qiagen) from various cell lines was used as template for cDNA synthesis (SuperScript II Reverse Transcriptase, Invitrogen). SAP30L gene-specific primers

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Abbreviations: GST, glutathione *S*-transferase; HDAC, histone deacetylase; kDa, kilodalton; SAP30L, Sin3A-associated protein 30-like; wt, wild-type

0014-5793/$32.00
doi:10.1016/j.febslet.2007.11.084

(5′-AGCACGGAGGAGGACAGCCGCGAA-3′ and 5′-TCAAGCTGCTTGCCACCCTCCGAT-3′, Proligo), located at the 5′ end and 3′ end of the coding sequence, were used for the PCR amplification of splicing isoforms with AmpliTaq DNA polymerase (Applied Biosystems). Amplified PCR products were analysed on 1% agarose gel, subcloned into a pCR®2.1-TOPO® vector (Invitrogen) and sequenced.

2.3. Cell culture, immunoprecipitation, Western blot analysis, and confocal microscopy

Human cervical carcinoma (HeLa), erythroleukemia (K-562), and promyelocytic leukaemia (HL-60) cells were cultured in RPMI1640 (Gibco) supplemented with penicillin–streptomycin antibiotics and 10% fetal bovine serum (FBS). Human acute myeloid leukaemia (OCI-AML3) cells were cultured in alpha-MEM (Gibco) supplemented with penicillin–streptomycin antibiotics and 20% FBS. Human embryonal kidney epithelial cells (HEK293T) were cultured and transient transfections, together with immunoprecipitation (IP), Western blotting (WB) and confocal microscopy were performed as previously described [10]. For immunoprecipitation, the agarose-conjugated primary antibodies used were c-myc (9E10; sc-40AC, Santa Cruz) or His (H-3; sc-8036AC, Santa Cruz), and for Western blotting, the primary antibodies against c-myc (9E10; sc-40, Santa Cruz) and actin (C2; sc-8432, Santa Cruz) and the antibody raised against the C-terminal human SAP30L peptide (VKS-peptide: ^{165}VKSNKSRLDQKSEGGKQLE^{183}) were used. The SAP30L antibody was a generous gift from Dr. Stephen Hall (AlphaGenix, California, USA). For immunoblot analysis of human tissues, INSTA-Blot® Human tissues ready-to-use membrane (Imgenex) was used and probed according to manufacturer's instructions. Detection was performed as previously described [10]. For confocal microscopy, the primary antibodies used were c-myc (A-14; sc-789, Santa Cruz) and nucleophosmin (32-5200, Zymed).

2.4. HDAC activity assay and transcriptional repression analysis

GST-pull-downs, HDAC activity measurements, and repression analyses were performed as described by Viiri and associates [10].

3. Results

3.1. Expression of SAP30L protein in human cells and tissues

To assess the expression of endogenous SAP30L protein, we used the polyclonal antibody hereafter called the VKS, raised against the C-terminal peptide. The ability of the VKS antibody to recognize SAP30L protein was first validated against glutathione-*S*-transferase (GST)-tagged recombinant SAP30L expressed in bacteria, as illustrated in Fig. 1A (see also http://www.alphagenix.com). GST-SAP30 fusion protein remained unrecognised, confirming the specificity of the VKS antibody (Fig. 1A). Transfected myc-his-tagged SAP30L migrates at around 31–32 kilodalton (kDa) and slightly faster compared to SAP30 [10]. This proportion correlates well with the calculated molecular weights of 20.8 kDa for SAP30L and 23.3 kDa for SAP30. Notably, in immunoblot analysis of human cancer cell lines, the 30 kDa protein was undetectable. Other proteins were detected, including a ubiquitous protein of around 18 kDa (Fig. 1B). The detected proteins were almost completely removed by pre-incubation of the antibody with the VKS peptide, as shown in Fig. 1B, but not with an irrelevant N-terminal SAP30L peptide (data not shown). In immunoblot analysis of a panel of human tissues, a protein of 30 kDa size could be detected and was strongly expressed in human brain and ovary and weakly in heart, small intestine, kidney, lung, skeletal muscle, stomach, and spleen (Fig. 1C). Several other proteins were also detected. To explore the possibility of proteolytic processing of SAP30L, a series of protease inhibitors were tested (PMSF, benzamidine, chymostatin, antipain, leupeptin, and pepstatin A), but none had effects on the immunoblotting profile elicited by the VKS antibody (data not shown). Based on these results, we propose that the 30 kDa protein probably represents the full-length SAP30L protein and other forms represent degradation products, splice variants or other post-transcriptionally modified forms of SAP30L.

3.2. Characterization of SAP30L isoforms

Prompted by the above immunoblotting data and previous Northern blotting results which suggested the presence of several mRNA forms [9], we investigated whether SAP30L mRNA could be alternatively spliced. For this purpose, we extracted RNA from several cell lines and performed RT-PCR using SAP30L-specific primers (Fig. 2A). In agarose gel electrophoresis, 1–4 separate DNA molecules could be identified and sequencing of them revealed three novel transcripts of SAP30L. Fig. 2B illustrates the structure of the SAP30L gene and the identified splice forms. The ΔEx2 splice variant is lacking the second exon. The ΔEx2Δ5aa form lacks, in addition to the second exon, also five amino acids from the third exon. The third splice form of SAP30L, ΔEx3,4, consists of exons 1 and 2, and has a spliced-in sequence from the intron between exons 2 and 3 with a frameshift mutation leading to a premature stop codon. All three splice variants have correct acceptor/donor sequences, as shown in Fig. 2B.

3.3. SAP30L isoforms differ in transcriptional repression capabilities, and associated HDAC activities

The ability of SAP30L isoforms to induce transcriptional repression was examined using Gal4DBD fusions. In line with our previous data, wild-type (wt) Gal4SAP30L fusion protein repressed the transcription of a luciferase reporter containing 14D promoter and Gal4 binding sites (5xGal4-14D-LUC) approximately 33-fold compared to Gal4 alone. The ΔEx2 splice form evinced a slightly reduced transcriptional repression activity compared to wt SAP30L (Fig. 3A). Surprisingly, the ΔEx2Δ5aa variant, which lacks a further five amino acids, had almost five times less repression activity compared to wt SAP30L. To further elucidate this, we created a mutant SAP30L protein lacking only these five residues (Δ109–113). Repression analysis confirmed the critical role played by these residues, showing markedly reduced repression capability with this mutant (Fig. 3A). The third splice variant, ΔEx3,4, which lacks the C-terminal Sin3A-binding region, showed only minor capability to repress transcription in this assay.

We have previously reported that SAP30L is associated with HDAC activity and identified HDACs 1–3 as putative binding partners for SAP30L [10]. We therefore next carried out pull-down experiments with GST-SAP30L isoforms from HEK293T nuclear lysates and measured the HDAC activity in the pull-down specimens, as shown in Fig. 3B. The ΔEx2, ΔEx2Δ5aa, and ΔEx3,4 splice forms pulled down markedly less of HDAC activity compared to wt SAP30L whereas Δ109–113 deletion mutant showed almost comparable HDAC activity to wt SAP30L.

3.4. SAP30L ΔEx3,4 variant is localized partly in the cytoplasm and does not bind Sin3A

SAP30L has previously been shown to be localized in the nucleoplasm and the nucleolus of tissue culture cells. The nu-