Alternative mRNA splicing of SAP30L regulates its transcriptional repression activity

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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 deacetylases. These modifications are catalyzed by 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.

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 (ΔEx2ΔSaa), 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) were generous gifts from D. Ayer (Salt Lake City, USA).

2.2. Identification of mRNA splice forms
RNA isolated (RNasey Mini Kit, Qiagen) from various cell lines was used as template for cDNA synthesis (SuperScript II Reverse Transcriptase, Invitrogen). SAP30L gene-specific primers 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. Histone deacetylase (HDAC) activity is a well-conserved mechanism for silencing gene expression [4]. Histones are typically deacetylated by a histone deacetylase complex which recruits HDAC complex to E2F transcription factors, leading to 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.
and 30. 2.3. Cell culture, immunoprecipitation, Western blot analysis, and confocal microscopy

Human cervical carcinoma (HeLa), erythroleukemia (K-562), and promyelocytic leukemia (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 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-40, Santa Cruz) or His (H-3; sc-8432, Santa Cruz) and actin (C2; sc-8432, Santa Cruz) and the antibody raised against the C-terminal human SAP30L peptide (VKS- peptide: 165VKSNKSDLQKSEGGKQLE183) were used. The SAP30L antibody was a generous gift from Dr. Stephen Hall (AlphaGenix, California, USA). For immunoblot analysis of human tissues, INSTA-Blot (AlphaGenix, California, USA) was used and probed according to manufacturer’s instructions. Detection was performed as previously described [10]. For confocal microscopy, the primary antibodies were used 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-SAP30L 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 ubiquitinous 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 (ΔEx9–113). 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 ΔEx9–113 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-
Fig. 1. Expression of endogenous SAP30L protein. (A) The specificity of the affinity-purified VKS peptide antibody was tested by immunoblotting of bacterially produced GST-SAP30L-fusion protein. As a control, GST-SAP30 fusion protein was used. (B) Immunoblot analysis of the indicated human cancer cell lines. Peptide inhibition was performed before immunoblotting using VKS peptide of SAP30L (see Section 2). Specific protein bands are indicated with *. (C) Immunoblot analysis of a panel of human tissues (20 µg total protein/lane).

Fig. 2. Identification of SAP30L mRNA splice forms. (A) RNA extracted from the indicated cell lines was used as a template for RT-PCR. The amplified PCR products were run on an agarose gel. Arrows indicate the major bands seen on gel electrophoresis. (B) Schematic structure of the SAP30L gene and the identified mRNA splice forms. Also depicted are the NLS and NoLS signals. GenBank Accession Nos.: wt SAP30L (AY341060), SAP30LΔEx2 (AI436556), SAP30LΔEx2Δ5aa (AI199517) and SAP30LΔEx3,4 (AI857594).
clear (NLS) and nucleolar localization signals (NoLS) were shown to direct the subcellular localization of SAP30L [9,10]. In confocal microscopy of HEK293T cells, ΔEx3,4 isoform was located partly in the cytoplasm and the nucleus but not in the nucleolus as demonstrated by double-staining with a nucleolar marker protein nucleophosmin (Fig. 4). Other natural isoforms displayed no evident changes in subcellular localization compared to wt SAP30L (Fig. 4). Notably, the ΔEx2 variant devoid of NLS signal was able to localize in the nucleus suggesting that the NoLS signal is sufficient in directing the nuclear localization in this context. An artificial deletion mutant lacking only five residues (Δ109–113) showed less of the nucleolar staining compared to wt protein (Fig. 4).

Finally, we explored whether SAP30L isoforms differed in their binding to corepressor Sin3A. Based on previous data [10] and the above repression data, one might conceive that the ΔEx3,4 variant is unable to associate with Sin3A. This was confirmed by coimmunoprecipitation experiments, as shown in Fig. 5. The other isoforms interacted with Sin3A comparably to wt SAP30L protein.

4. Discussion

Here we report the identification of three novel mRNA splice forms of SAP30L and describe putative isoforms also
at protein level. These isoforms reveal the critical role of the C-terminus of SAP30L in regulating its transcriptional repression capability. Furthermore, we describe a specific five-residue deletion mutant with severely reduced repression activity. Our data confirm that most of the repression activity of SAP30L is probably carried out by Sin3A and HDACs. The findings shed light on the molecular mechanisms regulating the transcriptional repression of Sin3A complex through SAP30L protein.

SAP30L is regulated at several levels. First, at the level of gene expression as shown by diverse expression of SAP30L mRNA in separate tissues ([9], http://genome.ucsc.edu) and by induction of expression after TGF-β-induced differentiation of T84 cells [9]; second, at the level of post-transcriptional modifications, as indicated by the production of alternative mRNA splice forms; third, at the translational/post-translational level, as suggested by strong expression of SAP30L protein in e.g. the brain and weak expression in the heart, and by a lack of 30 kDa protein in the tested cancer cell lines. All this points to the need for tight regulation of SAP30L and here we have begun to address this issue and have indicated that different isoforms of SAP30L evince functionally divergent properties. We isolated three novel transcripts of SAP30L which lacked some portions of the full-length protein. This led to varied reduction of transcriptional repression through SAP30L, as demonstrated by Gal4-SAP30L-fusion proteins and the luciferase reporter system. The most significant drop in repression activity was seen with the△Ex3,4 splice variant, where only minor activity could be detected. The △Ex3,4 variant was also unable to associate with Sin3A. This is consistent with our previous data showing the inability of Sin3A to interact with a C-terminally deleted SAP30L [10], and suggests that the SAP30L repressive function is mostly carried out by the Sin3A corepressor complex. Although △Ex3,4 splice form showed dramatically reduced repression activity, it still retained moderate association with HDAC enzymes, highlighting the functional importance of Sin3A itself in the complex. Intriguingly, these results also suggest that the repressional function of Sin3A corepressor complex is regulated not only at the level of sequence-specific transcriptional repressors, but also by associated proteins such as SAP30L.

In addition, we found markedly reduced transcriptional repression of SAP30L after deletion of five residues in the middle of the protein. These five residues lie between the NLS and the NoLS signals but the mutation leaves them intact. We found no evident changes in the binding to Sin3A whereas subcellularly this mutant was rarely seen in the nucleolus. Although the

Fig. 4. Subcellular localization of SAP30L splice variants by confocal microscopy. HEK293T cells were transiently transfected with the myc-histagged SAP30L proteins and immunostained using anti-myc antibody. Double-staining was performed by a nucleolar marker protein nucleophosmin (NPM). Cells in the right panel were treated for 10 h with 10 μM proteasome inhibitor MG132, which has been reported to cause accumulation of SAP30L in the nucleolus [10].
matter remains unproven, deletion of these amino acids could unfold the three-dimensional structure of the protein and thus change its ability to localize properly and mediate repression. Another intriguing possibility is that the deletion of these five residues influences the protein–protein interactions of SAP30L. These hypotheses remain to be tested in future experiments.

In immunoblotting experiments, we found several proteins specifically recognized by the SAP30L antibody. In gel electrophoresis, a small ubiquitous band migrated at around 18 kDa and thus slower than expected in order to represent the D
Ex2
 or D
Ex2
D
5aa isoforms (D
Ex3
,4 does not contain the VKS epitope). It may have arisen from non-specific degradation or specific proteolytic cleavage of the full-length SAP30L protein though we failed to prove this. The large proteins detected by the VKS antibody could represent novel SAP30L splice variants. In this respect, it is noteworthy that we have previously reported the presence of a large transcript of around 6 kb in Northern blotting using SAP30L-specific probe [9]. Furthermore, in chromosome 5 and upstream of SAP30L gene, there is a gene for N-acetylgalactosaminyltransferase 10 (GALNT10) which is predicted by Geneid program to generate a fused mRNA with SAP30L. This prediction is, however, awaiting experimental proof. Finally, since SAP30L is associated with a protein complex down-regulating gene expression, the absence of wt SAP30L protein in cancer cells could indicate it a crucial role in controlling transcription of genes driving e.g. cell proliferation.

In summary, this study adds a new piece of information regarding the molecular mechanisms influencing the transcriptional repression of the Sin3A corepressor complex through SAP30L, and highlights the intricacy of the regulatory mechanism involved in DNA transcription.

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References


Fig. 5. Association of SAP30L splice variants with Sin3A. The indicated myc-his-tagged SAP30L proteins and myc-tagged Sin3A (pCS2 + MT-Sin3A) were cotransfected into HEK293T cells, immunoprecipitated with agarose-conjugated anti-his antibody and immunoblotted with anti-myc or anti-VKS antibodies. Sin3A and SAP30L are indicated with arrows and the IgG light chain is marked with an *.