

Association of yeast SAP1, a novel member of the 'AAA' ATPase family of proteins, with the chromatin protein SIN1

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Abstract The yeast SIN1 protein is a nuclear protein that together with other proteins behaves as a transcriptional repressor of a family of genes. In addition, *sin1* mutants are defective in proper mitotic chromosome segregation. In an effort to understand the basis for these phenotypes, we employed the yeast two-hybrid system to identify proteins that interact with SIN1 in vivo. Here, we demonstrate that SAP1, a novel protein belonging to the 'AAA' family of ATPases, is able to directly interact with SIN1. Furthermore, we show, using recombinant molecules in vitro, that a short 27 amino acid sequence near the N-terminal of SIN1 is sufficient to bind SAP1. Previous experiments defined different domains of SIN1 that interact with other proteins and with DNA. The C-terminal domain of SIN1 was shown to be responsible for interaction with a protein that binds the regulatory region of *HO*, a gene whose transcription is repressed by SIN1. The central 'HMG1-like region' of SIN1 binds DNA, while the N-terminal of SIN1 can bind CDC23, a protein that regulates chromosome segregation. These data, taken together with the results presented here, suggest that SIN1 is a multifunctional chromatin protein that can interact with a number of different proteins that are involved in several different cellular functions.

Key words: Chromatin structure; Protein-protein interaction; Transcriptional repression; AAA family of ATPases; *SIN1/SPT2*; SWI/SNF complex; *Saccharomyces cerevisiae*

1. Introduction

SIN1/SPT2 is a non-histone chromatin component in yeast that was first described as a negative transcriptional regulator of a family of genes [1–3]. Detailed study has indicated that SIN1 functionally interacts with the C-terminal domain of RNA polymerase II [4], that it is antagonized by components of the SWI/SNF complex [1], that it can interact with a protein that binds the *HO* promoter [5], and that under certain conditions it can act as a positive transcriptional regulator [2]. Analysis of the predicted amino acid sequence revealed an internal amino acid domain that bears sequence and potential structural similarity to mammalian HMG1 [2,6,7]. SIN1 is able to bind DNA non-specifically [2], and we have recently shown that the HMG1-like domain alone is able to bind

DNA in vitro [8]. Interestingly, *sin1* mutants are also defective in proper segregation of chromosome III but not chromosome V during mitosis [2]. We have shown in parallel work to that described in this paper, that the N-terminal of SIN1 is capable of interaction with CDC23 [9], a protein known to control chromosome segregation during mitosis [10–12].

Here we extend our previous studies to help explain the biochemical basis for the disparate phenotypes of *SIN1*. We used the yeast two-hybrid system [13–15] as a method to identify proteins that directly associate with SIN1. We demonstrate that a short 27 amino acid sequence near the N-terminus of SIN1 is able to directly interact with a novel protein we have termed SAP1 (SIN1 associating protein). The predicted amino acid sequence of SAP1 indicates that it is a member of the 'AAA' (ATPase associated with diverse cellular activities) family of proteins.

2. Materials and methods

2.1. Plasmids and yeast strains

Plasmid pBTM116/SIN1 was constructed by subcloning the *EcoRI* fragment from pGEX-3X/SIN1 [16] containing the SIN1 coding region into the *EcoRI* site of pBTM116 [15]. This construct contains the complete LexA protein coding sequence fused in frame to the entire SIN1 coding sequence under the control of the yeast ADH1 promoter. pBTM116/SIN1 was transformed into yeast strain CTY10-5d (*MATa ade2, trp1-901, leu2-3,112, his3-200, gal4, gal80, URA3::lexA op-lacZ*) [15]. The pGAD libraries [14] were screened using the yeast two-hybrid system. Plasmid pGAD-21 carrying about a 5 kb insert was isolated from the libraries and was found by partial sequencing to contain an open translational reading frame (ORF) that was contiguous with the GAL4 protein ORF in the library vector. Computer BLAST nucleotide searches against yeast genomic databases using our cloned DNA as a 'probe' showed that our clone contained the DNA sequences coding for amino acids 214–897 of a putative yeast protein (accession number U18796, reverse translation of nucleotides 26050–28743). This putative protein had been deposited in the SWISSPROT database with the accession number P39955 (termed YEN7). Plasmid pSAP1 was constructed by PCR amplification of most of the *SAP1* coding region (covering amino acids 236–897) using pGAD-21 as a template, and its insertion between the *EcoRI* and *XbaI* sites of pBLUESCRIPT. The primers used for the PCR amplification of *SAP1* were: forward primer GGAATTCATGATAGATTGAC-TAATGACGAGG, reverse primer GCTCTAGAGGCTAGAATAT-TATTTATTATTACC.

Plasmids coding for the GST/SIN1 fusions illustrated in Fig. 2 were synthesized by amplification of portions of the *SIN1* gene in a PCR reaction, and subsequent subcloning into the vector pGEX-3X as described [5,9,16]. The peptide spanning amino acids 39–65 of SIN1 was translated from a nucleotide sequence amplified from primers GCGGATCCACATAAGAGACGAAGATCCTG and CCGAATT-CACCTAATTCCAAGGCCAC.

2.2. DNA sequencing

DNA sequencing of plasmids isolated from the pGAD libraries was performed by the Biological Services of the Weizmann Institute of Science using an Applied Biosystems 373 DNA Stretch Sequencer and dideoxy Taq terminators.

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Abbreviations: AAA, ATPases associated with diverse cellular activities; ORF, open reading frame; UAS, upstream activating sequence

2.3. Oligonucleotide synthesis

Oligonucleotides were synthesized by Biotechnology General (Kiryat Weizmann, Rehovot, Israel).

2.4. Coupled transcription/translation

Radiolabelled [³⁵S]methionine-labelled SAP1 protein and its derivatives were synthesized in a coupled T3 transcription/translation system (TNT (Promega)).

2.5. In vitro binding assay

The in vitro binding assay was done as described [9].

2.6. Computer analysis

Computer analysis was carried out using the Sequence Analysis software package of the Genetics Computer Group.

3. Results

3.1. The two-hybrid system indicates that SIN1 and SAP1 can interact in vivo

The coding region of *SIN1* was subcloned downstream and in frame to LexA in plasmid pBTM116 [15]. The resulting plasmid was transformed into yeast strain CTY10-5d [15] which contained a LexA operator in a UAS-less CYC promoter upstream of a lacZ gene. Western blot analysis, using anti-SIN1 antibodies directed against extracts from this transformed yeast strain, confirmed high levels of expression of the LexA/SIN1 fusion protein (data not shown). The *S. cerevisiae* pGAD libraries [14] were transformed into the yeast expressing the LexA/SIN1 fusion protein. About 350 000 colonies were screened for β-galactosidase activity, of which 12 were positive. Nine of the library plasmids recovered caused β-galactosidase expression independent of the LexA/SIN1 fusion and were discarded. The inserts in the three remaining plasmids were partially sequenced and compared against the GenBank/EMBL sequence databases. One plasmid was found to contain an in frame GAL4 activating domain/CDC23 fusion encompassing the entire CDC23 coding region [9]. Another plasmid contained a novel protein that we termed SAP1 (for SIN1 associating protein).

3.2. SIN1 and SAP1 can interact in vitro

To determine whether the SIN1 and SAP1 proteins can interact directly, we synthesized SIN1 and a portion of SAP1 in vitro and asked whether they could bind each other. To do so, the nucleotides of *SAP1* encoding amino acids 236–897 were amplified from the cloned library DNA using PCR and then subcloned into pBLUESCRIPT. The primer was designed such that it included a methionine in frame with the *SAP1* coding sequence immediately downstream of the T3 promoter. Radiolabelled SAP1 peptides were synthesized from this plasmid in a coupled transcription/translation system. SIN1 was expressed in *E. coli* as a fusion protein to glutathione-S-transferase (GST). GST/SIN1 was bound to glutathione-agarose beads and was mixed with the radiolabelled SAP1 peptides. After washing, the proteins were eluted from the beads with glutathione, separated by SDS-PAGE and autoradiographed. As can be seen in Fig. 1 (left lanes), the radiolabelled SAP1 bound the GST/SIN1 fusion protein while it did not bind GST alone. Importantly, while a number of polypeptides were synthesized in the TNT reaction having a smaller molecular weight than the full length SAP1 molecule, only a subset of the larger polypeptides including the presumptive full length SAP1 peptide bound the GST/SIN1.

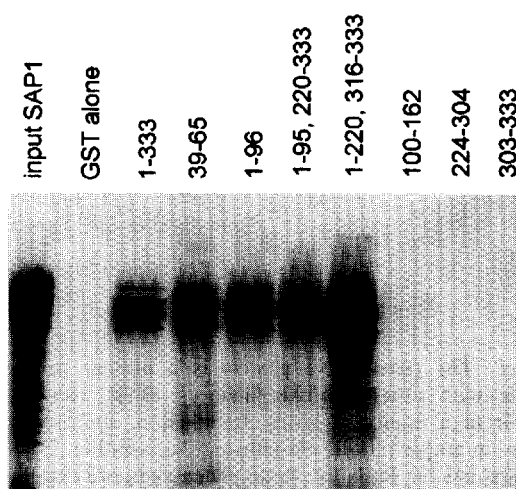


Fig. 1. Association between SIN1 and SAP1 proteins in vitro. GST or GST/SIN1 fusion proteins were immobilized on glutathione-agarose beads and incubated with [³⁵S]methionine-labelled SAP1 peptides. After washing, bound proteins were eluted with glutathione, resolved by SDS/PAGE, and analyzed by autoradiography. The multiple bands represent partial SAP1 molecules that result from premature transcriptional and translational termination, internal transcriptional initiation, and RNA and protein degradation. The label 'input SAP1' on the left-most lane indicates the product of the transcription/translation reaction that was applied to the GST or GST/SIN1 columns. The remaining lanes depict the SAP1 transcription/translation products that bound GST alone, or GST/SIN1 with the amino acid ranges of SIN1 denoted in the label.

This in vitro experiment demonstrates that the SIN1 and SAP1 molecules can interact with each other directly, without an intermediary yeast protein.

3.3. A peptide near the N-terminal of the SIN1 molecule is sufficient to interact with SAP1

Previous work has defined several hypothetical functional domains in SIN1 based on genetic, structural and biochemical considerations (Fig. 2) [2,5,7,17]. We therefore synthesized GST/sin1 fusions that contain portions of the SIN1 molecule based on the functional domains that have been suggested, bound them to glutathione-agarose beads, and asked whether the radiolabelled SAP1 molecule would bind the partial SIN1 molecule. As seen in Fig. 1, only peptides that contained amino acids 39–65 of SIN1 bound the SAP1, while GST/SIN1 molecules containing solely GST or the 'HMG domain' (amino acids 100–162), the 'acidic domain' (amino acids 224–304), or the C-terminal (amino acids 303–333) of SIN1 did not bind SAP1 at all.

3.4. SAP1 is a member of the 'AAA' family of ATPase proteins

The nucleotide and protein sequences of SAP1 are shown in Fig. 3. It is interesting and significant that the ORF of SAP1 is followed by eight consecutive stop codons. 100 base pairs following the last stop codon there is a string of 34 consecutive thymine residues interrupted by only two other nucleotides. It is unlikely that transcription extends beyond this sequence.

Comparison of the SAP1 sequence to protein databases shows that SAP1 contains significant homology to the AAA family of ATPases [18]. Conserved motifs of this family are denoted in the figure. Fig. 4 shows an alignment between SAP1 and other members of the AAA family that are closely

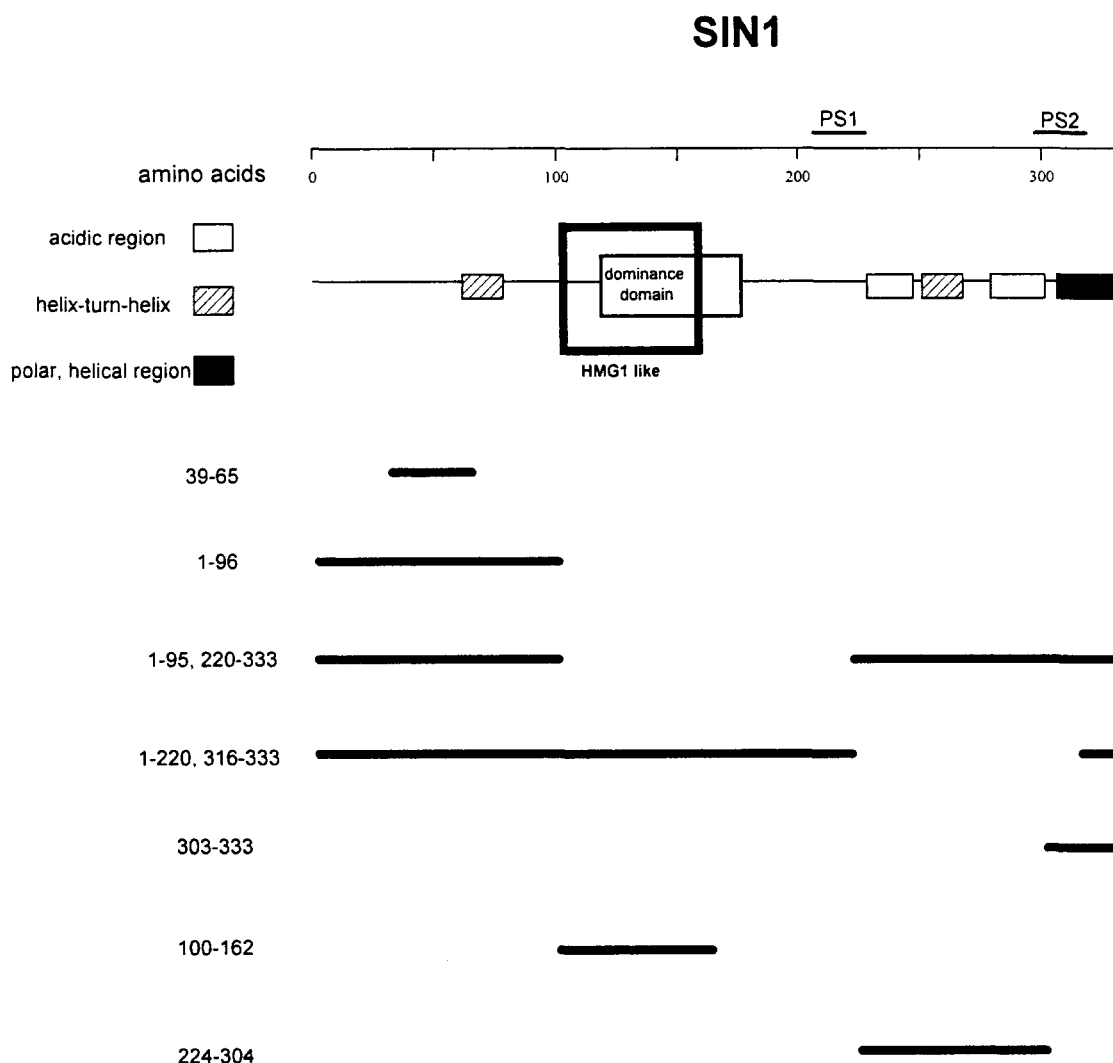


Fig. 2. Salient features of the SIN1 molecule. The functional regions are those defined by Lefebvre and Smith [17]. PS1 and PS2 represent the synthetic peptides against which the SIN1 antibodies were raised. The lower part of the figure illustrates the portions of SIN1 that were produced as GST fusion proteins.

related. As can be seen in the figure, regions that are conserved between the different proteins are conserved in SAP1 as well.

4. Discussion

SIN1 is known to participate in the transcriptional regulation of a family of genes including *HO*, *INO1* and the glucocorticoid receptor when expressed in yeast [2,4,16,17,19]. It is thought to primarily act as a transcriptional repressor antagonizing the SWI/SNF complex whose function is to remodel chromatin as a prerequisite for specific gene expression [20–27]. In earlier work, we showed that the C-terminus of the SIN1 molecule interacts with a protein that binds the *HO* promoter [5,16]. We suggested that the transcriptional regulation of *HO* and other genes by SIN1 may be mediated by sequence specific DNA binding proteins such as the one we identified. More recently, we have reported that the N-terminus of SIN1 interacts with CDC23 [9], a protein known to be required for the metaphase to anaphase transition during mitosis. In this study we have identified a novel putative ATPase

we termed SAP1, that interacts both in vivo and in vitro with SIN1.

Molecular mapping of the SIN1 molecule has revealed that the central 'HMG1-like' domain binds DNA [8], the C-terminal can interact with a protein that binds the *HO* promoter, and the N-terminal can interact with CDC23. Now we show that a short 27 amino acid peptide near the N-terminal of SIN1 is sufficient to bind SAP1. Taken together, these data support the notion that SIN1 is involved in several cellular functions, by serving as an anchor in the chromatin for molecules that are involved in controlling more than one process. This affords the cell the possibility to conserve valuable space in the chromatin by assigning several roles to the same molecule. Future experiments will address the question of whether the different proteins that interact with SIN1 compete for one another for a SIN1 binding site, whether they cooperatively bind, and if they participate in a common protein complex. It is noted that CDC23 and SAP1 both bind a domain of SIN1 that is near the N-terminus of SIN1. While SAP1 binds a SIN1 peptide ranging from amino acids 39–65 with the same affinity as a peptide ranging from amino acids 1–96,

TCCTTCCTTTCACTCGTGGGTTTTACCGAGTATTATATCGCACTGTGACACTATGGATTACAAAGAGCCATCATATTTAACGAGATTAAACA
 M D S Q R S H H I L T R L T K 15
 AGATACGGAGAGACCTCAACACCGTTAACCGACTTTACAGAACTTTACAGTAGAATTGCCAATGAGACAATATATTACTGAACCTAGAAGAAAA
 15 I R R R P Q Q P L T D F T E L Y S R I A N E T I Y Y L N L E E K K 48
 ACGATATAAGAAAGCATTGCAAGGATGGAAAGCGCTTACCACTGATGTACTATTCAACAGACATTGATAGAGCATAACTACCCAAATACTCAATCATAC
 48 R Y K E A L Q G W K A L T T D V L F K Q T L I E H N Y P N T Q S Y 81
 ACGAAGATGAAGTTAGTTACAGAACGTTATCGAGAATTGTACCAAGAGCGTTATGCATTGAAAAGAGTCAAAAAATTGGTCCGAGAGAGCGCTG
 82 T K D E V S L Q N G I R E L Y H K S V M H L K R V K K L V R E E P A 115
 CTCACGGAATGATATGCGGTATCCAAAACATACACTAATCATAGCTCATCTTTCACAGGCTCTACTGAACACCTCTGTATCCAAATGGTTCGCGG
 115 P R N D M P S S K T Y T N H S S S F T R S T E P P P V F Q M V P G 148
 AAGAATGATGAAACATTAAGAAATAGAAATGATGCGGCTATAAAACCGCATACTCCAAACCTCTACTATCATATGGAACAGCAGCATCGATAAAA
 148 R M M K T L R N R N A C G Y K T A Y S N P S L S S Y G N S T S I K 181
 CGCGGAGAGATGGGAGAACATTAGATAAATTTGTACCTCTTAAGCCTTTATCCAAATGCAAGCAGACAGCACAAGAACCTATTGAGCACAATG
 182 R G E D A E N I R V N F V P S K P L S N N A S R Q H K N P I E H N D 215
 ATCCCTCACTTAAGAAGAACTGAACCTGACTCAGATAAATATATCTCAGAGCAATATTGATAGATTGACTAATGACGAGGATGATCAGATGTCGG
 215 P L L K K E T E L Y S D K Y I S E P I L I D L T N D E D D H D V P 248
 CATTTTAAAGGACACACGTTTTGATGAAGAGAAAGTGTGTTGAGTTGATGTTGCGATTACTATGATAATTTCCGAGAGTACGAGTAGAA
 248 I L K G H N V F D E E E S D G F E F D V S D Y Y D N F S E V D V E 281
 GAAGAAGAAGAAAAAGGAGAGACGAGTATAAAACGTTAGAAGCAATCAACAGCAATGTCTGACTTGTCTGTACTTCTCTACGTCAGTA
 282 E E E E E K E E R R R I K T L E A I Q Q Q M S D L S V T S S T S S N 315
 ATAAGAGTGAAGTTCTTCAAAAATGTTCCGAGTATCATACAGTCCCTGCGACTACCGGCGCTGCTCTTCTTCAATACCTCCACCTCTTATT
 315 K S V S S S E N V P G S C I Q S L P T T A P A L P S L P P P P L L 348
 AAATGTCGATAGAGCGTCCAGCACTGGGCGCTTAAAGCCACATAGTTTGAAGAACTTCTACTACTATGGATTATCAAGATAGGAATCTCAATATCA
 348 N V D R A S S T G A L K P H S L E T S T T M D S S K I R N P Q I S 381
 AAATTAATGAAGATAATCAGTACCATACTTAAAGGTACCAATCAACGCCACCTTATCACTAAATCAACGCCAACATTATTACAGATCAAAAA
 382 K L M K N N H V P Y L K G T K S T P T L I T K S T P T F I T R S K S 415
 GTAATACAAAACCAATCATCAATCCAAATGATCGTCTCGACCTCTCTCTGACTGTTCGAAATTCAGTAATCAAAAACCCAAACCGCTGCAATGGC
 415 N T K P I I K S N A S S P T S S L T V P N S V I Q K P K T A A M A 448
 TGTGAAGAGTCTGAACAGCAAAAAGGTTGCGAGTAACCGCGCATTAACACACTACGAAGAAGAGTCAACCCCATTTTGAATCCAAAACGGCGAAGTC
 448 A K R V L N S K K V A S N P A L N T T K K S H P I L K S K T A K V 481
 CCAATTCAGTTCGAAGAAAGAGTAGCCATCTTCAGACCTGTAAGTAACCAAAACCATCTCGCATGGTGCATCCAGAAATGAAGGCCATCGA
 482 P N S S S K K T S S H P S R F V S N S K P Y S H G A S Q N K K P S K 515
 AAAATCAAAACGCTCTATGATAAACGAATGAAATAACAGCACAATAAATCGTCTCCAAAATAGAAGATGTTGGAACAGAGATGCCAC
 515 N Q T T S M S K T N R K I P A Q K K I G S P K I E D V G T E D A T 548
 TGAATGCCACTTCCCTAAATGAGCAAGAGAGAGCGCTGAATAGACAAGAAAGTCTGAGGAGATTAGAGATGAATATTGATAGTTTACAA
 548 E H A T S L N E Q R E E P E I D K K V L R E I L E D E I I D S L Q 581
 GGTGTAGATAGCGAGCTGCAAGCAAAATTTTCCGGAATCGTATGACAGGAGATGAAGTTTATGAGATGATTTGCTGGTTTAGAAGTGCAAAAT
 582 G V D R Q A A K Q I F A E I V V H G D E V H W D D I A G L E S A K Y 615
 ATTCTTTGAAGGAAGCAGTTGTCTATCCGTTTTTGGAGCAGACTTATCAGGGGTTACGTGAACAGTCAGGGGATGCTCTTATTGGACCCAGG
 615 S L K E A V V Y P F L R P D L F R G L R E P V R G M L L F G P P G 648
 TACAGGTAAACAAATGCTAGCGAGAGCTGTAGTACAGAGTGCAGCTCCACCTTTTCTCTATTAGTGCCTCCAGTTTGACATCTAAATACTTGGGTGA
 648 T G K T M L A R A V A T E S H S T F F S I S A S S L T S K Y L G E 681
 AGTGAATAATAGTGAAGGCACTATTGCAATTTGCCAAAAATGTCACTCTATAATATTGTTGATGAATTTGACTTATCATGGGTAGTAGGAATA
 682 S E K L V R A L F A I A K K L S P S I I F V D E I D S I M G S R N N 715
 ACGAAATGAAATGAGTCAAGCGAAGGATAAGAAATGAATTTCTGTTCACTGGTATCTCTGTCAGCGCAGCGGCTGTTCAACAAAGTAACAC
 715 E N E H E S S R R I K N E F L V Q W S S L S S A A A G S N K S N T 748
 TAACAATCTGACACCAATGGCGACGAAGATGATACAAGGTTACTGGTACTTCCCGGACAAACTTACCATGCTTATTGATGAGCTGCCAAGGAGAAGA
 748 N N S D T N G D E D D T R V L V L A A T N L P W S I D E A A R R R 781
 TTTGTGAGAGACATATATCCCATTACAGAGGACGAGACGACAGCTTCAATTTAAGAGCTTCTTCCCATCAAAAGCACAGCTTAACCGAATCAG
 782 F V R R Q Y I P L P E D Q T R H V Q F K K L L S H Q K H T L T E S D 815
 ATTTTGAAGTGGTAAAGATTACTGAGGCTATTGAGGAGTATACGCTCTTACGCAAGGATGCTGCCATGGGCGCCTACGAGATCTGGGTGA
 815 F D E L V K I T E G Y S G S D I T S L A K D A A M G P L R D L G D 848
 TAAGTTATTAGAAACAGAGAGGAGATGATAAGACCATAGGCGCTTGTGATTTTAAAGAACGTTTAGTGATATCAAGCCCTCTGTATCCAGGACGGA
 848 K L L E T E R E M I R P I G L V D F K N S L V Y I K P S V S Q D G 881
 TTAGTGAGTACGAAAGTGGCTTCACAATTCGATCATCAGGTTCAATGATGATGATAATAATAATAATAATAATAATAATAATAATAATAATAATA
 882 L V K Y E K W A S Q F G S E G S * * * * * 897
 ATCTAGCCTAGCTGGACAACTGTGTGCACATAAGCGGATGATAAATAATGTCTGCATAATGTCACCTTTCTTTTTTTTTTTTTTTTTCTTTT
 TTTGACTACTTAGTTTGTAAACAAATTAATATTAGCGTAAACTATTACTAACTAATCCATGACTGGTGTAGGTAGTCTTACCCAGACCGCTCTTCT

Fig. 3. Sequence of *SAPI* and its translation product. The arrow indicates the start of the sequence that was isolated from the pGAD library. The boxed regions indicate the regions of homology with other members of the 'AAA' family that are depicted in Fig. 4.

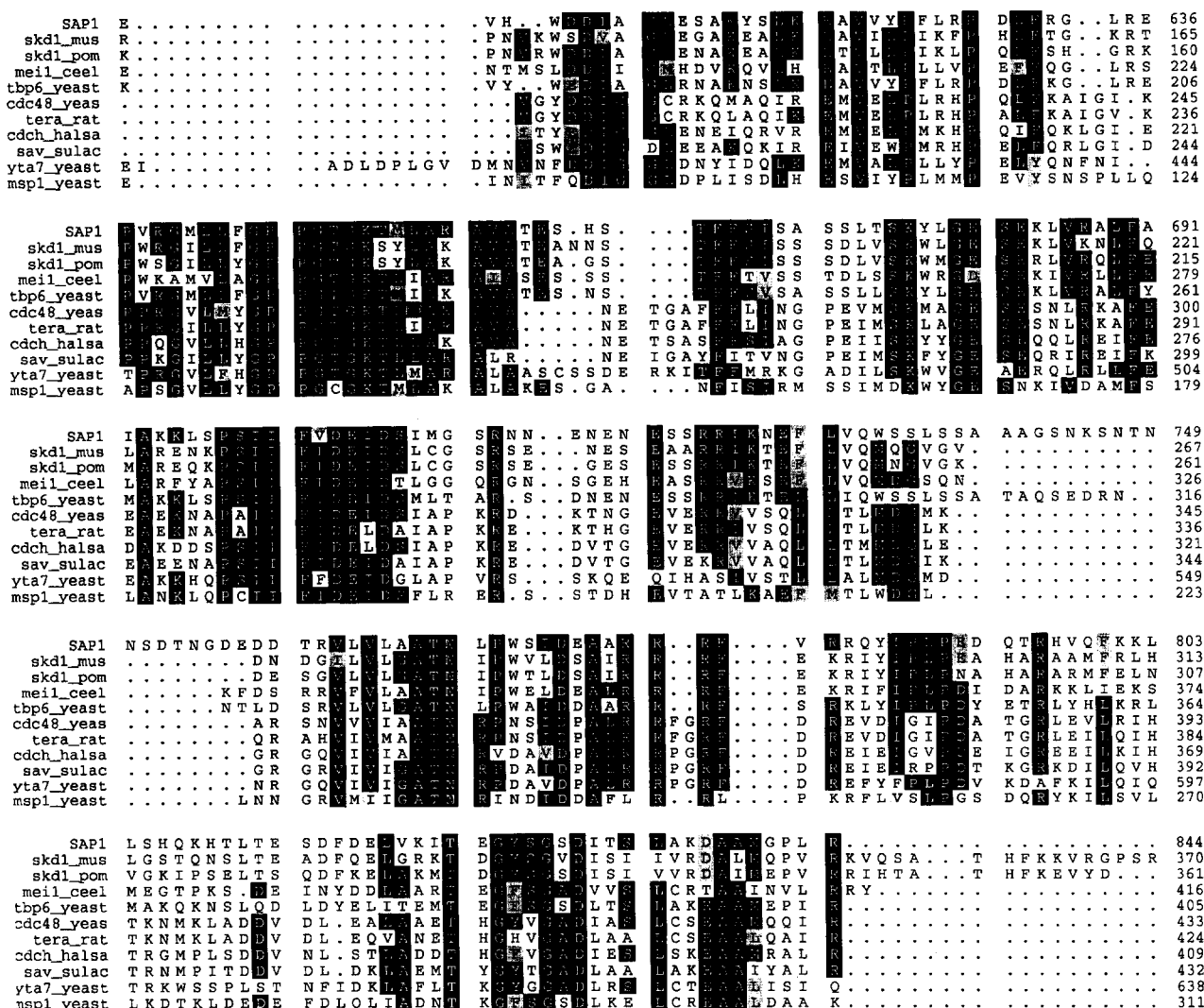


Fig. 4. PILEUP and PRETTYBOX (Genetics Computer Group) alignment of SAP1 and other members of the 'AAA' family. We display only the portion of the alignment that contains significant homology between the sequences. The respective accession numbers of the sequences are: skd1_mus, p46467; skd1_pom, q09803; meil1_ceel, p34808; tbp6_yeast, p40328; cdc48_yeast, p25694; tera_rat, p46462; cdch_halsa, p46464; sav_sulac, q07590; yta7_yeast, p40340; msp1_yeast, p28737.

CDC23 binds the shorter peptide less tenaciously than the longer peptide (unpublished).

The fact that SAP1 appears to be a member of the 'AAA' family [18] is especially intriguing since members of this family are associated with the 26S proteasome (e.g. YTA2 and YTA5 [28] and SUG1 [29] in yeast) that is necessary for proper chromosome segregation, and with transcriptional activators (e.g. human TBP1 (Tat binding protein) [30]). It has been suggested by comparing members of this family that it may be possible to distinguish proteasomal from transcriptional proteins by their estimated isoelectric points (4.5–5.6 versus 8–9.5, respectively) [31]. The estimated isoelectric point of SAP1 is 9.76 (using the ISOELECTRIC program (Genetics Computer Group)) suggesting that SAP1 is more likely to be involved in transcription rather than in a proteasomal subunit. Interestingly, SIN1 functionally, if not directly, interacts with the SWI/SNF complex that contains SWI2/SNF2. SWI2/SNF2 is an ATPase/helicase [32] that is thought to be central to the chromatin remodelling function of the entire SWI/SNF complex. Recently, another ATPase unrelated to the SWI/

SNF complex has been identified in *Drosophila* [33,34], which is also involved in chromatin remodelling. These data support the notion that there may be parallel protein complexes that are differentially utilized, to modulate the structure of varying portions of the chromatin. It remains to be seen whether SAP1 is a protein that participates in one of these complexes.

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