

# SAG/ROC2/Rbx2/Hrt2, a Component of SCF E3 Ubiquitin Ligase: Genomic Structure, a Splicing Variant, and Two Family Pseudogenes

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## ABSTRACT

We have recently cloned and characterized an evolutionarily conserved gene, **Sensitive to Apoptosis Gene (SAG)**, which encodes a redox-sensitive antioxidant protein that protects cells from apoptosis induced by redox agents. The SAG protein was later found to be the second family member of ROC/Rbx/Hrt, a component of the Skp1-cullin-F box protein (SCF) E3 ubiquitin ligase, being required for yeast growth and capable of promoting cell growth during serum starvation. Here, we report the genomic structure of the SAG gene that consists of four exons and three introns. We also report the characterization of a SAG splicing variant (SAG-*v*), that contains an additional exon (exon 2; 264 bp) not present in wildtype SAG. The inclusion of exon 2 disrupts the SAG ORF and gives rise to a protein of 108 amino acids that contains the first 59 amino acids identical to SAG and a 49-amino acid novel sequence at the C terminus. The entire RING-finger domain of SAG was not translated because of several inframe stop codons within the exon 2. The SAG-*v* protein was expressed in multiple human tissues as well as cell lines, but at a much lower level than wildtype SAG. Unlike SAG, SAG-*v* was not able to rescue yeast cells from lethality in a *ySAG* knockout, nor did it bind to cullin-1 or have ligase activity, probably because of the lack of the RING-finger domain. Finally, we report the identification of two SAG family pseudogenes, *SAGP1* and *SAGP2*, that share 36% or 47% sequence identity with ROC1/Rbx1/Hrt1 and 30% or 88% with SAG, respectively. Both genes are intronless with two inframe stop codons.

## INTRODUCTION

**C**ELL CYCLE PROGRESSION is precisely regulated by timely synthesis and degradation of specific regulatory proteins. The major protein degradation pathway involves the ubiquitin/proteasome system. Ubiquitination of a target protein involves a multistep, well-defined set of reactions catalyzed by a cascade of enzyme families, including ubiquitin-activating enzyme E1, ubiquitin-conjugating enzymes E2, and ubiquitin ligases E3. Ubiquitin is first activated via binding to E1 through a thioester bond between a cysteine residue at the active site of E1 and the C-terminal glycine (G76) of ubiquitin. Activated ubiquitin in the E1-ubiquitin complex is then transferred to E2 that also forms a thioester bond between its active-site cysteine residue and G76 of ubiquitin. Finally, through the action of E3, ubiquitin is covalently attached to the target protein through an isopeptide bond between G76 of ubiquitin and the  $\epsilon$  amino

group of an internal lysine residue of the target protein. Through multiple reactions, a chain of ubiquitins is covalently attached to the substrates to form polyubiquitinated conjugates that are rapidly recognized and degraded by the 26S proteasome (Ciechanover, 1998).

One of the well-defined E3 ubiquitin ligase complexes in eukaryotes that control cell cycle progression of  $G_1 \rightarrow S$  is SCF complex, consisting of Skp1, cullins/Cdc53, and E-box proteins (Bai *et al.*, 1996; Skowyra *et al.*, 1997). The SCF complexes with a newly identified RING-H2 protein, ROC/Rbx/Hrt (Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Tan *et al.*, 1999), to constitute an active E3 ubiquitin ligase that promotes ubiquitination and degradation of cyclin-dependent kinase inhibitors, such as p27 in mammals and Sic1 in yeast (Carrano *et al.*, 1999; Feldman *et al.*, 1997; Kamura *et al.*, 1999; King *et al.*, 1996; Skowyra *et al.*, 1997; Sutterluty *et al.*, 1999; Tsvetkov *et al.*, 1999).

We have also recently cloned and characterized an evolutionarily conserved RING finger protein, SAG (Sensitive to Apoptosis Gene), that is redox inducible and protects cells from apoptosis induced by redox agents (Duan *et al.*, 1999; Sun, 1999, 2000; Swaroop *et al.*, 1999). Sequence comparison has revealed that SAG is the second member of the Rbx/ROC/Hrt family (ROC2/Rbx2/Hrt2). The SAG/ROC/Rbx is required for yeast cell growth (Kamura *et al.*, 1999; Ohta *et al.*, 1999; Swaroop *et al.*, 2000), can promote cell growth and S phase entry on serum starvation if overexpressed (Duan *et al.*, 2001), and can inhibit tumor cell growth if repressed by antisense transfection (Huang *et al.*, 2001). Furthermore, two SAG deletion variants were recently identified, and the RING-H2 domain was found to be required for apoptosis protection (Sun, 1999). Here, we defined the genomic structure of the human SAG gene and characterized a SAG splicing variant (SAG-v) and two pseudogenes (SAGP1 and SAGP2) of the SAG/ROC/Rbx/Hrt family.

## MATERIALS AND METHODS

### Computer database search

The database was searched using BLAST (tblastn) from NCBI (Altschul *et al.*, 1997). The sequences were from the 113 release of GenBank (Benson *et al.*, 2000) and were clustered using the LEADS™ software from Compugen (Tel Aviv, Israel).

### Cloning of SAG-v and SAGP1

Epitope-tagged SAG-v and SAGP1 were cloned by RT-PCR with muscle or heart mRNA (Clontech) as the template (Sun *et al.*, 1993b). For FLAG-tagged SAG-v, the primers used were SAG-Bam-Flag01: 5'-CGCGGATCCGCCACCATGGACTACAAGGACGACGATGACAAGGCCGACGTGGAA-GACGGA-3' and SAG-VAR-Xh02: 5'-CCGCTCGAGCTACCGATGGTCATCCAGACA-3'. For HA-tagged SAG-v, the primers were SAG-HA01: 5'-CGCGGATCCGCCACCATGTATCCATATGATGTTCCAGATTATGCTGCCGACGTGGAAGAC-3' and SAG-VAR-Xh02. For HA-tagged SAGP1, the primers were SAG3-HA-Bam03: 5'-CGCGGATCCGCCACCATGTATCCATATGATGTTCCAGATTATGCTTTGTGGTTAATAACTGTGC-3' and SAG3-Xh02: 5'-CCGCTCGAGTCACA AAACAGTTAAGCTTGATGG-3' that amplifies a cDNA fragment encoding 84 amino acids (translating from the second methionine). To validate SAGP1 EST sequences, RT-PCR direct sequencing (Sun *et al.*, 1993a) was performed with primers SAG3-Bam1A: 5'-CGCGGATCCGGTAAAGTGTCTGTGC-3' and SAG3-Xh02. To simultaneously amplify both SAG and SAG-v, the primers SAG-Bam-Flag01 and SAGV.02: 5'-GTAGGGCACTGGATTACAAG-3' were used. The PCR products were cloned into pcDNA3 and sequenced.

### Northern and Western blot analysis

Northern analysis was performed as described previously (Sun *et al.*, 1992). To determine SAG expression in transformed yeast cells, Western blot analysis was performed as described (Swaroop *et al.*, 2000). Briefly, yeast cell extracts were pre-

pared by vortexing the yeast with glass beads in the presence of urea/SDS lysis buffer supplemented with protease inhibitors. Cell extracts were then subjected to PAGE and transferred to a nylon membrane. The membrane was probed with HA-peroxidase antibody (Boehringer Mannheim) followed by incubation with enhanced chemiluminescence Western blotting detection reagents (Amersham) and autoradiography.

### Complementation, yeast sporulation, and dissection

The HA-tagged human SAG and SAG-v were cloned into the yeast expression vector p424-TRP for complementation studies. The constructs were transformed into the heterozygous knockout strain (ySAG/ySAG::Kan) using the YeastMaker Transformation System from Clontech. Expression of SAG and SAG-v was detected by Western blotting using anti-HA antibody. The clones that expressed exogenous protein were sporulated and dissected as described previously (Swaroop *et al.*, 2000).

### Transfection, metabolic labeling, and extract preparation

Transient transfection studies were carried out in human 293T cells grown in DMEM (GIBCO/BRL) containing heat-inactivated FBS (GIBCO/BRL). Cells were cotransfected with pcDNA3 vectors expressing either HA-SAG or HA-SAG-v and cullin-1 plasmid by the calcium phosphate method (Sun *et al.*, 1997). At 48 h post-transfection, cells were metabolically labeled for 2 h with Easy Tag Express (<sup>35</sup>S) Protein labeling mix (100  $\mu$ Ci/ml) from NEN. Cell pellets were resuspended in 0.2 ml of buffer A (10 mM Tris HCl, pH 7.4; 10 mM NaCl, 0.5% NP40, 1 mM PMSF, antipain 2  $\mu$ g/ml, leupeptin 2  $\mu$ g/ml) and lysed by sonication. An equal volume of buffer B (20 mM Tris HCl, pH 7.4; 1M NaCl, 0.2% NP40, 1 mM PMSF, antipain 2  $\mu$ g/ml, and leupeptin 2  $\mu$ g/ml) was added, and the resulting mixture was rocked for 1 h at 4°C prior to centrifugation at 100,000  $\times$  g at 4°C for 1 h.

### Immunoprecipitation

The supernatant liquid prepared above was rocked with HA antibody (HA-7; Sigma) and protein agarose beads (40  $\mu$ l; Upstate Biotechnology) for 1 h at 4°C. The beads were washed sequentially three times with 0.5 ml of buffer C (buffer A and B mixed in equal volumes) and two times with 0.5 ml of buffer D (25 mM Tris HCl, pH 7.4; 1 mM EDTA, 0.01% NP40, 10% glycerol, and 50 mM NaCl). Bound proteins were released by boiling the beads in the presence of 20  $\mu$ l of SDS loading buffer. Half the reaction mixture was used for SDS-PAGE, followed by autoradiography.

### Ubiquitin labeling and Ub ligation assay

The assay was performed as described previously (Swaroop *et al.*, 2000). Protein kinase-ubiquitin (PK-Ub), prepared as a fusion protein with a protein kinase C recognition site (LRRASV), was kindly provided by Dr. Z. Pan at Mount Sinai School of Medicine, New York. The PK-Ub (7  $\mu$ g) was first labeled with <sup>32</sup>P-ATP in a 20- $\mu$ l reaction mixture containing 20 mM Tris HCl, pH 7.4; 12 mM MgCl<sub>2</sub>, 2 mM NaF, 50 mM NaCl, 25  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]-ATP, BSA 0.1 mg/ml, and 1 U of cAMP kinase (Sigma). The reaction mixture was incubated

at 37°C for 30 min, followed by heat inactivation of the kinase at 70°C for 3 min. Immunoprecipitated samples, prepared as described above, were added to a Ub ligation reaction mixture (30  $\mu$ l) that contained 50 mM Tris HCl, pH 7.4; 5 mM MgCl<sub>2</sub>, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 1  $\mu$ g of <sup>32</sup>P-Ub, 60 ng of E1 (Affiniti), and 300 ng of mouse Cdc34 (E2; kindly provided by Dr. Z. Pan). The reaction mixture was incubated at 37°C for 1 h with constant shaking. The reaction mixture was then combined with 20  $\mu$ l of 4 $\times$  Laemmli loading buffer, boiled for 3 min, and subjected to 12.5% and 7.5% SDS-PAGE analysis, followed by autoradiography.

## RESULTS

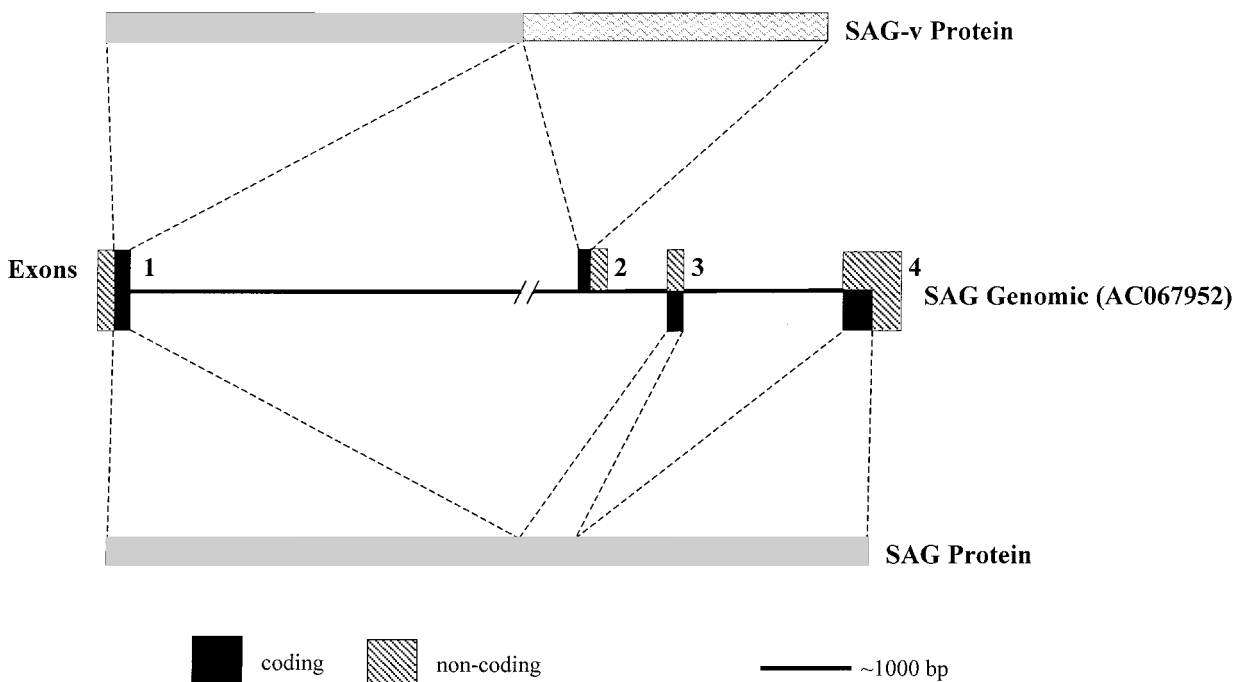
### Genomic structure of human SAG/ROC2/Rbx2/Hrt2 gene

We have previously reported the cloning of a 754-bp cDNA fragment (nt 1–342; the ORF; nt 343–754; the 3' UTR) that encode SAG, a redox-inducible protein and the second member of ROC/Rbx/Hrt family (Duan *et al.*, 1999; Ohta *et al.*, 1999; Swaroop *et al.*, 2000). Using this fragment as a Northern probe, we determined that the size of SAG mRNA is about 800 bp (Duan *et al.*, 1999). The SAG gene was mapped to chromosome 3q22–24 (Duan *et al.*, 1999). To define the genomic structure of SAG, we searched the GenBank database with this 754-bp cDNA fragment (Accession No. AF092878) as the bait. An 192-kb chromosome 3 genomic clone, consisting of 35 unordered contigs (Accession No. AC067952.4), was shown to contain the

SAG gene. Comparison of SAG genomic and cDNA/protein sequences revealed that the wildtype SAG consists of three exons (exons 1, 3, and 4) (Fig. 1; also see below). Exon 1 encodes the first 59 amino acids, whereas the rest of the sequence, including the RING-finger domain, is encoded by the exons 3 and 4 (Fig. 1). The exon–intron boundary sequences are shown in Table 1, and all introns meet the GT-AG rule, beginning with the GT and ending with the AG. The exact length of intron 1 cannot be determined because of a gap of unknown length in the sequence. Recently, SAG was also cloned by others in a yeast two-hybrid screening and found to be a casein kinase II-binding protein (Son *et al.*, 1999). This SAG clone (Accession No. AF164679) consists of 836 bp with an additional 24 nt located at the 5' end of the ATG and mapped to the same exon as the 175 residues downstream from the ATG (exon 1). Thus, on the basis of SAG mRNA size, we have analyzed the nearly full-length of SAG cDNA and confirmed the SAG chromosomal DNA sequences.

### Identification and cloning of a human SAG variant (SAG-v)

The SAG/ROC/Rbx/Hrt family has two known members (Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Swaroop *et al.*, 2000; Tan *et al.*, 1999) that show high sequence similarity with Apc11, a component of the anaphase promoting complex (APC) (Yu *et al.*, 1998; Zachariae *et al.*, 1998). To identify other potential SAG family member(s), we searched a clustered EST database with known family members as the queries. A cluster of three ESTs (AA442424, AA749029, and N95727) gave rise to a SAG variant (SAG-v) that contains a



**FIG. 1.** SAG genomic and protein structure. The exons are indicated by the solid boxes with their respective numbers. Introns are indicated by the thin line. The double slashes indicate the location of a gap between contigs. The hashed areas indicate regions that are transcribed but not translated. The shaded boxes represent the ORF of SAG (**bottom**) and SAG-v (**top**) proteins. The projection of protein domains onto their corresponding exons is indicated. Also indicated are the C3H2C3 RING finger residues.

TABLE 1. EXON-INTRON BOUNDARY SEQUENCES OF *SAG/ROC2/Rbx2/Hrt2*

Exons	Intron-exon-intron junctions	SAG-v	AC067952.4
1	..... CAGGTGATGGgtaagcgctg	1-175	<36333-36507
2	ttttttacagATGAGGGAAT..... TTCTGTGTCAgtaagtatgg	176-439	189836-190099 reverse
3	ttacttttagATGCCTGTCT..... GACTGTGTGgtaagtgtgta	440-487	189187-189234 reverse
4	tttctttcagTGGTCTGGGG.....	488-end	187398->187584 reverse

The exon sequences are shown in upper case, whereas the intron sequences are in lower case. Only the partial sequences of each exon and intron adjacent to the splice sites are shown. The conserved intron (starting sequence GT and ending sequence AG) is underlined. Because the exact 5' and 3' ends of the SAG-v mRNA are not defined, < and > symbols are used to indicate that the sequence continues beyond the indicated numbering. Sequence positions in AC067952.4 are relative to the total clone numbering. Exons 2-4 are listed in reverse order from exon 1 because they fall within a separate contig of the AC067952 clone.

264-bp insertion at nucleotide 177 of the *SAG* ORF (Fig. 2A; underlined). The ORF of SAG-v predicts a protein of 108 amino acids with the first 59 amino acids identical to SAG and the remaining 49 amino acids a novel sequence at the C terminus. Because of inframe stop codons, the entire RING-H2 finger domain is not translated (Fig. 2B). Sequence comparison between SAG-v and the *SAG* genomic clone revealed a 100% match of a 264-bp insertion sequence with the genomic clone (not shown), indicating it belongs to an additional exon, not present in wildtype *SAG*. Thus, the genomic structure of *SAG* consists of four exons and three introns. The SAG-v consists of all four exons, whereas the wildtype *SAG* contains exons 1, 3, and 4 with exon 2 spliced out. Shown in Figure 1 is the protein structure of SAG-v and SAG and their corresponding encoding exons. The coding sequences for SAG-v is encoded by exons 1 and exon 2, whereas that for SAG is encoded by exons 1, 3, and 4 (bottom part of the figure). The entire ORF of SAG-v was cloned by RT-PCR using mRNA from human muscle and heart as the templates and was confirmed by DNA sequencing. The SAG-v sequence has been deposited in GenBank with the Accession Number AF312226.

#### Identification of two potential pseudogenes of *SAG/ROC/Rbx/Hrt* family

Similar EST database mining identified a cluster of two ESTs (AI023768 and AA99464) that predicts an ORF of 84 amino acids (translated from the second methionine), termed *SAGP1* (see below). Figure 3 shows a pile-up comparison among *SAG* family members. Five of the eight conserved C3H2C3 residues are preserved in *SAGP1*, and the other two substitutions (H → R, C → T) seen in SAG-p are also seen in some RING-finger proteins with degeneracy (Boddy *et al.*, 1994; Borden and Freemont, 1996). Thus, in *SAGP1*, the functional RING-finger domain appears to be conserved, and this clone may represent a third member of the *SAG/ROC/Rbx/Hrt* family. Direct RT-PCR sequencing of human heart mRNA using primers derived from the ESTs revealed an ORF of 112 codons with two inframe stop codons between the first and the second methionine (Fig. 3). The overall sequence identity of this clone is 36% with *ROCI* and 30% with *SAG*. Because disruption of an ORF by stop codons is reminiscent of a pseudogene, this clone was named *SAGP1* (potential pseudogene of

the *SAG/ROC/Rbx/Hrt* family). Furthermore, a high-throughput genomic (HTG) database search revealed that *SAGP1* maps to human chromosome 5 (Accession No. AC012636). The genomic sequence analysis showed that the *SAGP1* gene is intronless, another characteristic of a pseudogene. As will be discussed below, *SAGP1* is not translated into protein in all expression vectors tested, further suggesting that it is a pseudogene of the *SAG/ROC/Rbx/Hrt* family.

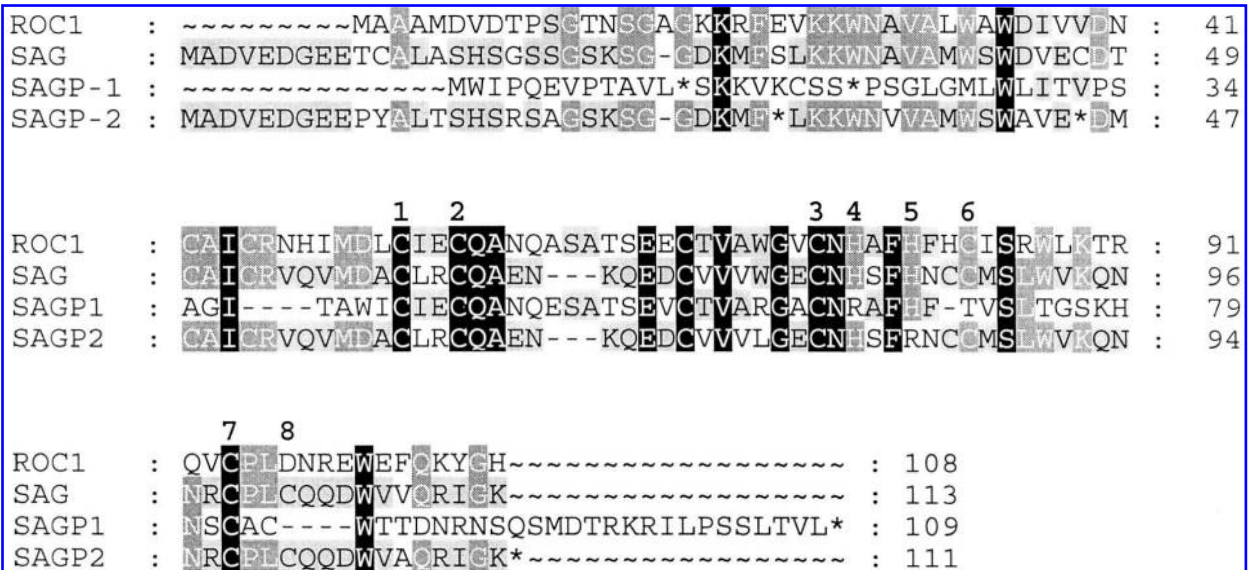
Similar HTG database searching revealed an additional pseudogene of the *SAG* family, designated *SAGP2* (Accession No. AC016923). This sequence mapped to chromosome 3 and shared 88% sequence identity with *SAG* and 47% identity with *ROCI*. As shown in Figure 3, all eight conserved C3H2C3 residues are preserved in *SAGP2*, except an H → R substitution at the fifth position of the RING domain. However, the *SAGP2* gene is intronless and contains two inframe stop codons, at positions 31 and 47, which well qualifies it as the second pseudogene of the *SAG/ROC* family.

#### Expression of SAG-v and SAGP1 in human tissues and cell lines

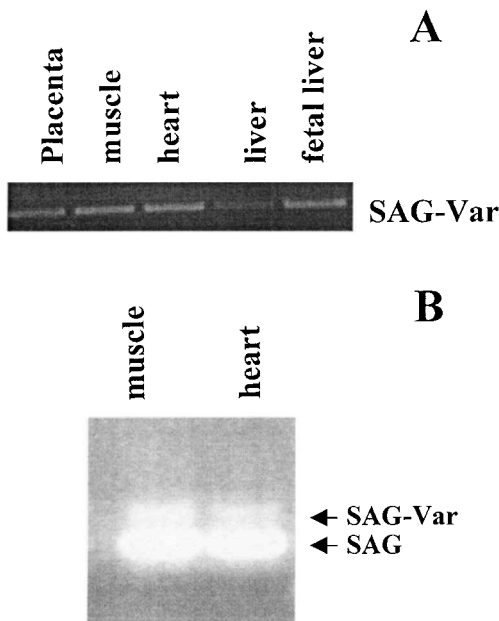
Using Image clone AA442424 (consisting of the sequence encoding a part of N-terminal SAG, 264-bp insertion sequence, and the sequence for the entire C-terminal RING domain) as a SAG-v probe for Northern analysis, we detected the expression of *SAG* as a 0.8-bp band, but no expression of SAG-v (expected to be 264 bp longer), in multiple human tissues (data not shown). The result suggested that expression of SAG-v, if any, is quite low. To increase detection sensitivity, we performed RT-PCR using the primers flanking the SAG-v ORF. As shown in Figure 4A, expression of SAG-v was detected in human placenta, muscle, heart, and liver (both adult and fetal). Expression of SAG-v was also detectable by RT-PCR in more than 20 human cell lines (data not shown).

To determine the relative expressions of *SAG* and SAG-v, we performed RT-PCR using a pair of primers flanking the entire ORF of *SAG*. If SAG-v is present as a 264-bp insertion variant, we should be able to detect a band with a size of 264 bp larger than wildtype *SAG*. Indeed, as shown in Figure 4B, in addition to a very abundant band corresponding to *SAG*, a faint band with a size corresponding to SAG-v was detected and confirmed by Southern blot analysis (data not shown).





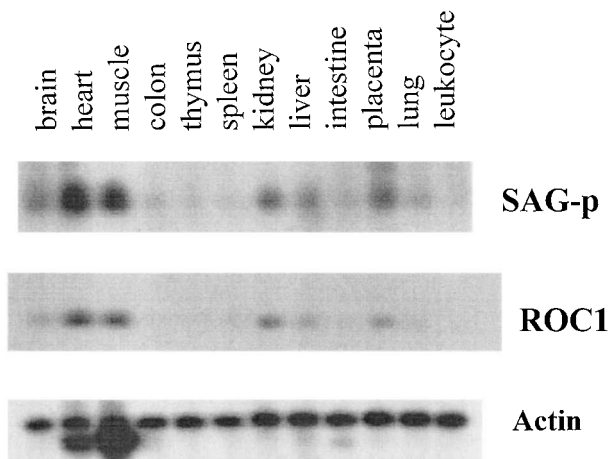
**FIG. 3.** Comparison of ROC1 and SAG/ROC2 with two SAG family pseudogenes, *SAGP1* and *SAGP2*. For *SAGP1*, two ESTs (AI023768 and AA699464) were identified through a database search using ROC/SAG as queries. The sequence shown here was confirmed by RT-PCR direct sequencing. For *SAGP2*, a genomic coding (AC016923) was identified through a HTG database search using a 754-bp SAG cDNA as a query. Translation was initiated from the first ATG for protein sequence comparison purposes. The numbers (1–8) above the amino acid codons indicate the residues forming the C3H2C3 RING finger domain. Identical residues in all four sequences are blackened, and identical residues in three sequences are shaded.



**FIG. 4.** Expression of SAG-*v* in human tissues. Total RNA from the indicated human tissues was subjected to RT-PCR using SAG-*v*-specific primers (note that the downstream primer is located within the insertion sequence) flanking the entire ORF (A) or specific primers for wildtype SAG that amplify both SAG and SAG-*v* (B), as detailed in Materials and Methods. The PCR fragments corresponding to SAG or SAG-*v* are indicated and were confirmed by Southern analysis.

#### *Unlike SAG, SAG-v does not rescue death phenotype induced by ySAG disruption*

We have previously shown that yeast *SAG* is essential for growth and that disruption of the gene leads to cell death, which can be rescued by wildtype human *SAG* but not by *SAG* mutants (Swaroop *et al.*, 2000). To test the potential rescuing activity of *SAG-v*, heterozygous *ySAG* knockout cells (*ySAG/ySAG::Kan*) were transformed with *SAG-v* cloned into the yeast expression vector p424 (carrying a tryptophan selectable marker) and also with wildtype *SAG* control. Transformants were selected by plating on tryptophan minus medium, and expression was confirmed by Western blot analysis with HA antibody. Figure 6A shows two individual clones that expressed wildtype *SAG* or *SAG-v*, respectively, at a compatible level. These clones were sporulated and dissected. Rescue of *ySAG::kan* cells should result in four viable haploids displaying three possible genotypes: wildtype (grown only in YPD medium), wildtype+h*SAG-v* plasmid (grown in YPD minus tryptophan), and *ySAG::kan* + h*SAG-v* plasmid (grown in YPD-G418-containing medium). The tetrad dissected from *SAG-v*-transformed *ySAG/ySAG::kan* cells gave rise to two viable haploids that grew only in YPD medium (Fig. 6B). This result strongly suggests that all viable haploids contain wildtype *ySAG*, an idea that was confirmed by PCR analysis (data not shown). Four viable haploids were seen in the tetrad dissected from wildtype *SAG*-transformed cells (Figure 6C) with the expected genotypes (Swaroop *et al.*, 2000). Thus, unlike wildtype *SAG*, *SAG-v* is unable to rescue the death phenotype induced by *ySAG* disruption. A similar yeast rescue assay was performed with a *SAGP1* construct (translated from the second methionine to give rise to an 84-amino acid protein). No ex-



**FIG. 5.** Expression of *SAGP1* and *ROC1* in multiple human tissues. A membrane blotted with 1  $\mu\text{g}$  of poly(A)<sup>+</sup> mRNA from indicated human tissues (ClonTech) was subjected to Northern analysis, probed with *SAG-p* cDNA (**top**), *ROC1* cDNA (**middle**), and  $\beta$ -actin (**bottom**) as described in Materials and Methods.

pression of *SAGP1* protein could be detected in transformed yeast cells.

#### *SAG-v* does not bind to *cullin-1* and has no ligase activity

We have recently shown that, like *ROC1/Rbx1/Hrt1*, *SAG* binds to *cullin-1* and has associated E3 ubiquitin ligase activity (Swaroop *et al.*, 2000). We also found that E3 ubiquitin ligase activity of *SAG/cullin-1* complex is required for the rescue of the death phenotype induced by *ySAG* disruption (Swaroop *et al.*, 2000). To further define the biochemical nature of *SAG-v*, we examined its potential ability to bind to

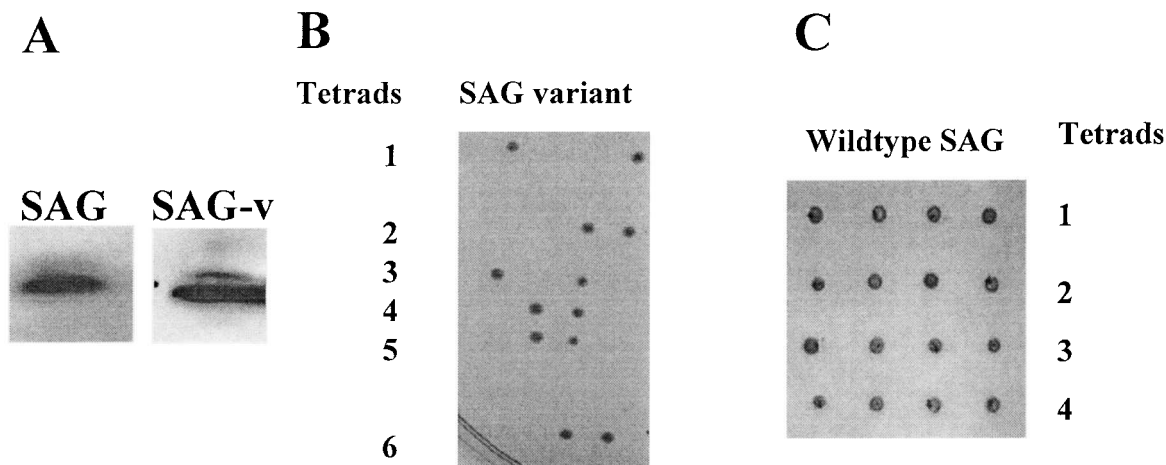
*cullin-1* and its E3 ubiquitin ligase activity. The *SAG-v* gene was cotransfected with *cullin-1*, along with vector/*cullin-1* and *SAG/cullin-1* cotransfection controls, into 293 cells, followed by <sup>35</sup>S-Met labeling, immunoprecipitation, and ligase activity assay. Because expression of *SAG-v* was much less than that of *SAG* after transfection, as measured by direct Western blot (data not shown), a fourfold excess of cell lysate from *SAG-v*-transfected cells was used for immunoprecipitation.

As shown in Figure 7A, the level of *SAG-v* and *SAG* proteins was comparable after adjustment for the input protein. Unlike *SAG*, which binds to *cullin-1* (lane 3, Fig. 7A), *SAG-v* was found not to bind with *cullin-1* (Fig. 7A). It is noteworthy that because of the fourfold excess of protein used in the *SAG-v* sample for the assay, some nonspecific binding bands were evident (lane 2). We also measured potential ligase activity of *SAG-v* compared with *ROC1* and *SAG*. As shown in Figure 7B, *ROC1* and *SAG* had E3 ubiquitin ligase activity and induced ubiquitin polymer formation (lanes 1 and 2), whereas *SAG-v* had very little, if any, such activity (lane 3). Thus, biochemically, *SAG-v* differs from wildtype *SAG*. The lack of ligase activity of *SAG-v* is most likely attributable to the absence of the RING-finger domain required for this activity.

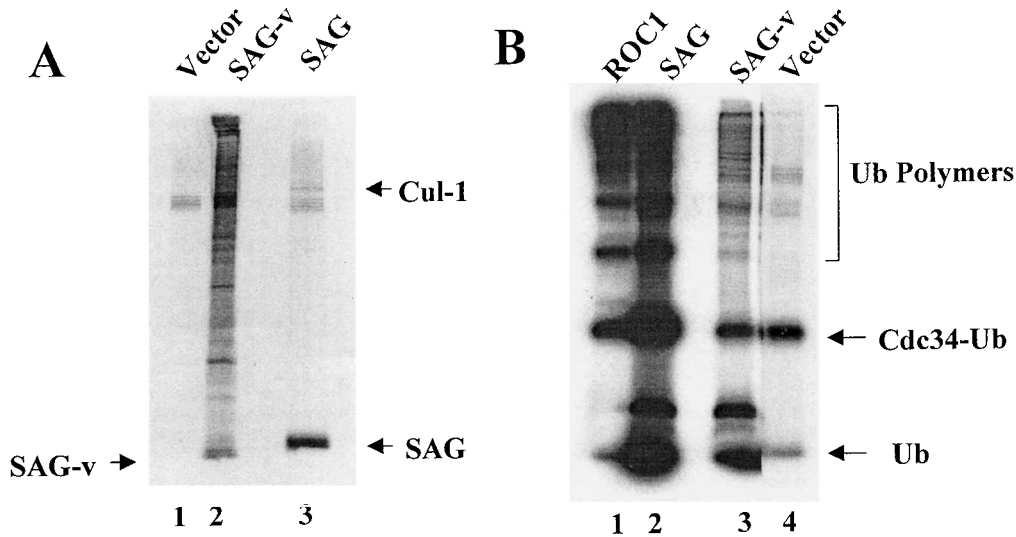
We have attempted to express *SAGP1* using constructs that would initiate protein translation from either the first methionine (containing inframe stop codons between two methionines; see Fig. 3) or the second methionine (encoding an 84-amino acid protein). Both *in vitro* transcription-coupled translation and *in vivo* human kidney 293 cell transfection assay showed no protein expression from either construct (data not shown), further confirming that *SAGP1* is a pseudogene of the *SAG/ROC* family. Similar analysis has not been performed with *SAGP2*.

## DISCUSSION

The *SAG* protein is evolutionarily conserved (for details, see review by Sun *et al.*, 2001). Two members of the



**FIG. 6.** *SAG-v* failed to rescue yeast death phenotype induced by *SAG* disruption. Heterozygous *ySAG* knockout cells (*ySAG/ySAG::kan*) were transformed with an *hSAG*- or *SAG-v*-expressing vector (p424) containing *TRP* as a selectable marker. Transformants were then selected on a *TRP*-minus plate. Several surviving clones were measured for *SAG* or *SAG-v* expression by Western blot. (A) Expression of *SAG* or *SAG-v* protein in two representative clones. These two clones were sporulated. Six independent tetrads from *SAG-v* (B) and four from *SAG* (C), as the control, were dissected and grown in YPD medium.



**FIG. 7.** SAG-v does not bind to Cul1 and has no ubiquitin ligase activity. **(A)** SAG and SAG-v expression and binding to cullin-1. HA-tagged SAG or HA-tagged SAG-v or the empty vector control was cotransfected with cullin-1 into 293 cells, followed by  $^{35}\text{S}$ -methionine labeling. Cell lysates were immunoprecipitated with HA-tag antibody, followed by gel electrophoresis and autoradiography. About fourfold excess of cell lysates was used for SAG-v to ensure an approximately equal level of SAG expression. **(B)** E3 ubiquitin ligase activity of ROC1 and SAG but not SAG-v. HA-tagged ROC1, wildtype SAG, SAG-v, or the empty vector control was cotransfected with cullin-1 into 293 cells, followed by  $^{35}\text{S}$ -methionine labeling. Cell lysates (with approximately equal amount of SAG and SAG-v protein) were immunoprecipitated with HA-tag antibody, followed by ligase activity assay, as detailed in Materials and Methods.

*SAG/ROCI* family have been cloned and characterized in humans. They are *SAG* (Accession No. AF092878) and *ROCI* (Accession No. AF142059) (Duan *et al.*, 1999; Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Tan *et al.*, 1999). Although *SAG* and *ROCI* share only 53% overall sequence identity, seven of eight cysteine/histidine residues that constitute the RING-finger domain are identical, and, importantly, they are functionally equivalent. Although uncharacterized, computer analysis revealed two additional family members: a hepatocellular carcinoma-associated RING-finger protein (Accession No. AF247565) and a hypothetical protein (Accession No. NP\_057560 or AF151048). The *SAG* and *ROCI* family is highly evolutionarily conserved across different species. A BLAST search identified two family members in mouse (Accession Nos. AF140599 and AF092877) with sequence identity of 100% with human *ROCI* and 96% with human *SAG* and four members in *Drosophila melanogaster* (Accession Nos. AAF45536, T13388, AAF47382, and AAF52694) with sequence identity ranging from 37% to 91%. In *Caenorhabditis elegans*, there are three members (Accession Nos. T27823, T29620, and T21802) with a sequence identity ranging from 35% to 71%. One family member was identified in the fission yeast *Schizosaccharomyces pombe* (Accession No. Z98977; 87% with *ROCI* and 59% with *SAG*), in budding yeast *Saccharomyces cerevisiae* (Accession No. NP\_014508; 66% with *ROCI* and 55% with *SAG*), in *Arabidopsis thaliana* (Accession No. CAB87200; 81% with *ROCI* and 51% with *SAG*); and in *Plasmodium falciparum* (Accession No. T18513; 68% with *ROCI* and 44% with *SAG*). In addition, *ROCI* is ho-

mologous to the APC11 subunit of the APC/cyclosome (*APC/C*) (Zachariae *et al.*, 1998). An Apc11 homolog is also found in yeast (Accession No. Z74056), *Drosophila* (Accession No. CAB63945), and possibly in *C. elegans* (Accession No. CAA86328).

Although *SAG* has been well characterized biochemically and biologically (Duan *et al.*, 1999, 2001; Huang *et al.*, 2001; Sun, 1999, 2000; Sun *et al.*, 2001; Swaroop *et al.*, 1999, 2000; Yang *et al.*, 2001), the genomic structure, splicing variant, and pseudogenes of *SAG* have not been reported. Here, we have defined the genomic structure of *SAG* that consists of four exons and three introns with exon 2 being spliced out in wildtype *SAG*. In addition to identify two pseudogenes, *SAGP1* and *SAGP2* of the *SAG* family, we have also characterized a *SAG* splicing variant, *SAG-v*, whose mRNA contains all four exons, but whose protein translation terminated at exon 2 because of several inframe stop codons. The *SAG-v* gene was found to be expressed at a much lower level than wildtype *SAG* in multiple human tissues. Because of its lack of a RING-finger domain, *SAG-v* has no E3 ubiquitin ligase activity and fails to complement yeast *SAG*. The biologic significance of *SAG-v* is unknown at the present time. It appears unlikely that *SAG-v* acts in a dominant-negative manner to inhibit *SAG* E3 ubiquitin ligase, as *SAG-v* does not bind to cullin-1. Thus, it would not compete with *SAG* for cullin-1 binding. However, we cannot exclude the possibility that *SAG-v* would compete with *SAG* for binding to other SCF components. Future study should be directed to identifying the *SAG-v* binding partners in an attempt to understand its biologic function.



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