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Forum Review

SAG/ROC/Rbx/Hrt, a Zinc RING Finger Gene Family: Molecular Cloning, Biochemical Properties, and Biological Functions

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ABSTRACT

The RING (really interesting new gene) finger proteins containing a characteristic C_3HC_4 or $C_3H_2C_3$ motif appear to act as E3 ubiquitin ligase and play important roles in many processes, including cell-cycle progression, oncogenesis, signal transduction, and development. This review is focused on SAG/ROC/Rbx/Hrt (sensitive to apoptosis gene/regulator of cullins/RING box protein), an evolutionarily conserved RING finger family of proteins that were cloned recently by several independent laboratories through differential display, yeast two-hybrid screening, or biochemical purification. SAG/ROC2/Rbx2/Hrt2 is expressed in multiple mouse adult tissues, as well as early embryos. In humans, both SAG and ROC1 are ubiquitously expressed at a very high level in heart, skeletal muscle, and testis. Expression of both SAG and ROC1 is induced by mitogenic stimulation. SAG is also induced by a redox agent in cultured cells, as well as in *in vivo* mouse brain upon ischemia/reperfusion. Structurally, *SAG* consists of four exons and three introns with at least one splicing variant and two pseudogenes. The SAG gene promoter is enriched with multiple transcription factor binding sites. Biochemically, SAG binds to RNA, has metal-ion binding/free radical scavenging activity, and is redox-sensitive. Most importantly, like ROC1, SAG/ROC2 binds to cullins and acts as an essential component of E3 ubiquitin ligase. Biologically, SAG is a growth-essential gene in yeast. In mammalian cells, SAG protects apoptosis mainly through inhibition of cytochrome *c* release/caspase activation, and promotes growth under serum deprivation at least in part by inhibiting p27 accumulation. Blocking SAG expression via antisense transfection inhibits tumor cell growth. Thus, SAG appears to be a valid drug target for anticancer therapy. Antioxid. Redox Signal. 3, 635–650.

THE RING FINGER PROTEINS: A GENERAL INTRODUCTION

THE ZINC RING (REALLY INTERESTING NEW GENE) FINGER PROTEINS are a newly defined gene family, containing a conserved motif, C_3HC_4, or its variant $C_3H_2C_3$ (also known as RING-H2), with the fifth cysteine substituted by a histidine residue (28, 70). This motif has four pairs of metal binding residues with a characteristic linear sequence of Cys-X_2-Cys-$X_{9–39}$-Cys-$X_{1–3}$-His-$X_{2–3}$-Cys/His-X_2-Cys-$X_{4–48}$-Cys-X_2-Cys, where X can be any amino acid, although there are distinct preferences for particular types of amino acid at a particular position (70). The RING finger motif binds to two zinc atoms per molecule in a cross-braced system, where the first and third pairs of cys-

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teine/histidine form the first binding site and the second and fourth pairs of cysteine/histidine form the other. Figure 1 shows a diagram of this RING finger motif.

Since its first recognition in 1991 (28), the family members have increased to >200 proteins from diverse eukaryotes, but interestingly not in any prokaryote protein (27, 70). The RING finger proteins showed a variety of biochemical properties (6, 7, 70), including DNA binding (53), transactivation (13, 65), or transrepression (69, 71); RNA binding (22, 46); metal-ion binding (20, 46, 66, 93); or protein binding (5, 63, 98). Most recently, almost all RING finger proteins tested are found to bind to E2 ubiquitin-conjugating enzymes and possess E3 ubiquitin ligases, which promote targeted protein degradation (27, 39, 40, 48, 52, 57, 95, 104). Biologically, the zinc RING finger proteins play important roles in many processes, including cell-cycle progression, oncogenesis, signal transduction, and development, among others (6, 7, 18, 70). Examples include Mdm2, an oncogene that regulates cell cycle through ubiquitination/degradation of p53 (23, 25, 26, 35, 36); BRCA-1, a breast cancer tumor suppressor (32, 61); PML, a promyelocytic leukemia protein that has a proapoptotic activity mediated through its RING domain (8); and baculovirus protein, p35, and its mammalian homologues of inhibitors of apoptosis (IAP) that inhibit apoptosis (16, 86, 91, 103). This review is only focused on ROC1/Rbx1/Hrt1 and SAG/ROC2/Rbx2/Hrt2, two members of an evolutionarily conserved RING finger family that biochemically bind to RNA and metal ions, have E3 ubiquitin ligase activity, and biologically regulate apoptosis and cell growth.

MOLECULAR CLONING AND CHARACTERIZATION

Cloning of SAG or ROC2/Rbx2/Hrt2

Reactive oxygen species (ROS), consisting of superoxide anion, hydrogen peroxide, hydroxyl radical, and organic peroxides, among others, are a group of very reactive, short-lived chemicals produced during normal metabolism or after an oxidative insult (75). They are highly cytotoxic by inducing DNA damage, lipid peroxidation, and protein degradation (75). ROS play an important role in induction of apoptosis (for reviews, see 9, 24, 60), either by causing direct cellular damage or by modifying cellular redox-sensitive molecules such as nuclear factor-κB (NF-κB) and p53 (79), known to regulate apoptosis (3, 44, 58, 83).

Apoptosis, characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, and DNA fragmentation (99), occurs in both embryonic cells during development and differentiated cells in adult tissues to maintain homeostasis under physiological conditions. Apoptosis is also widely involved in many pathological conditions. The common diseases associated with increased apoptosis include AIDS, ischemic injuries, and neurodegenerative disorders, whereas diseases associated with decreased apoptosis include autoimmune disorders and cancers (88). Mech-

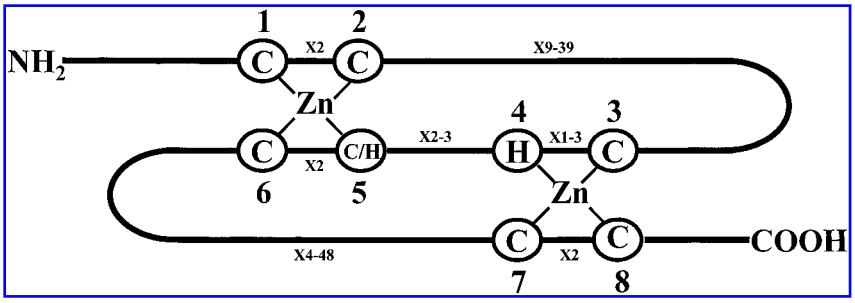


FIG. 1. A schematic structure of classical zinc RING finger motif found in many biologically important proteins. Shown in the C_3CH_4 or $C_3H_2C_3$ RING finger motif that binds to two zinc atoms in a cross-braced arrangement. The X stands for any amino acid, although some preferences at particular positions do exist. Note that the figure was not drawn to scale.

anism-driven anticancer drug discovery requires identification and validation of the molecular targets. Genes that confer tumor cells resistant to apoptosis would be ideal targets for cancer therapy. To identify such genes, we first established a p53-ROS-apoptosis cell model in which p53 is activated by 1,10-phenanthroline (OP; an agent that disturbs cellular redox balance by chelating transition metal ions), followed by cell death via apoptosis (78, 83). The choice of the p53 pathway was made because p53 is a key molecule in apoptosis regulation (44, 47) and dysfunctional p53 is detected in many human cancer cells (33, 34) that become resistant to anticancer agents (54, 55).

To identify genes responsive to OP-induced apoptosis in this model, we employed the mRNA differential display technique (50, 81) and used limited sets of the primers to screen ~1,200 genes. Two OP-inducible genes were cloned. The first gene is glutathione synthetase (76), an enzyme involved in the last step of glutathione synthesis (59) and previously unknown to be inducible by redox agent (76). The second gene is *SAG* (sensitive to apoptosis gene), a novel zinc RING finger protein, consisting of 113 amino acids. The protein is localized in both the cytoplasm and nucleus of the cells, and the gene is mapped onto human chromosome 3q22-24 (20). Recently, SAG was also identified in a yeast two-hybrid screening as a substrate of casein kinase II by binding to its regulatory (β) subunit (74). Casein kinase II is a Ser/Thr kinase that has many substrates and is implicated in cell division and proliferation (64).

Cloning of ROC1/Rbx1/Hrt1

Cell-cycle progression is precisely regulated by timely synthesis and degradation of cell-cycle regulatory proteins. The major protein degradation pathway involves the ubiquitin/proteasome system. Ubiquitination of a target protein involves multistep enzymatic reactions catalyzed by a cascade of enzymes, including ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase 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-terminus glycine (G76) of ubiquitin. Activated ubiquitin in E1–ubiquitin complex is then transferred to E2, which also forms a thioester bond between its active-site cysteine residue and G76 of ubiquitin. Finally, ubiquitin is covalently attached to the target protein through an isopeptide bond between G76 of ubiquitin and the ϵ amino group of an internal lysine residue of the target protein, catalyzed by E3. Through the multiple runs of the reactions, ubiquitin is covalently attached to the substrates to form polyubiquitinated conjugates that are rapidly recognized and degraded by the 26S proteasome (15).

One of the well defined E3 ubiquitin ligase complexes in eukaryotes that control cell-cycle progression is the SCF complex, consisting of Skp1, cullins/Cdc53, and F-box proteins (1, 73). SCF complex is mainly involved in G1 $\rightarrow$ S progression, but also plays a role in the other phases of the cell cycle (19, 85). In a search for additional component(s) in SCF complex that is required for its *in vitro* reconstituted ubiquitination activity, a RING-H2 protein was identified as a human cullin-1 binding protein in a yeast two-hybrid screening (62); as a novel component of SCF complexes purified from HeLa cells (87); as a fifth subunit of purified von Hippel–Lindau (VHL)/elonginC/elonginB/cullin-2 complexes (42); and as a specific component of Skp1 and cullin-1 immunoprecipitates (72). This protein was named ROC for regulator of cullins (62), Rbx for RING box protein (42), or Hrt (72). ROC/Rbx/Hrt consists of 108 amino acids with a RING finger domain at the C-terminus. The family has two members: ROC1 and ROC2. Direct sequencing comparison revealed that SAG is ROC2. Thus, redox inducible SAG turns out to be a component of SCF E3 ubiquitin ligase. For the sake of simplicity, this review will refer to SAG for SAG/ROC2/Rbx2/Hrt2 and ROC1 for ROC1/Rbx1/Hrt1.

SAG and ROC1 belong to an evolutionarily conserved gene family

At the present time, only two members of the SAG/ROC1 family have been cloned and characterized in humans: SAG (accession no. AF092878) and ROC1 (accession no. AF142059)

(20, 42, 62, 72, 87). Although they only share 53% overall sequence identity, seven of eight cysteine/histidine residues that constitute the RING finger domain are identical, and they are functional equivalent (see below). Blast search revealed potentially two additional family members: a hepatocellular carcinoma-associated RING finger protein (accession no. AF247565) and a hypothetical protein (accession no. NP_057560 or AF151048). In addition, there are at least two intronless pseudogenes (accession nos. AC016923 and AC012636) localized in chromosome 3 or 5, respectively, and one SAG splicing variant, being identified and characterized most recently (85a). The SAG and ROC1 family is highly evolutionarily conserved across different species. Blast search identified two family members in mouse (accession nos. AF140599 and AF092877) with sequence identity of 100% to human ROC1 and 96% to human SAG. Four members in *Drosophila melanogaster* (accession nos. AAF45536, T13388, AAF47382, and AAF52694) are revealed 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 member was identified in the following species: (a) fission yeast *Schizosaccharomyces pombe* (accession no. Z98977, 87% to ROC1 and 59% to SAG); (b) budding yeast *Saccharomyces cerevisiae* (accession no. NP_014508, 66% to ROC1 and 55% to SAG); (c) *Arabidopsis thaliana* (accession no. CAB87200, 81% to ROC1 and 51% to SAG); and (d) *Plasmodium falciparum* (accession no. T18513, 68% to ROC1 and 44% to SAG). Shown in Fig. 2 is the sequence comparison of SAG (A) and ROC1 (B) family members among different species with the highest sequence homologue from each species. In addition, ROC1 is homologous to the APC11 subunit of the anaphase-promoting complex/cyclosome (APC/C) (105). APC11 homologue is also found in *yeast* (accession no. Z74056), *Drosophila* (accession no. CAB63945), and possibly in *C. elegans* (accession no. CAA-86328).

Expression of SAG family genes

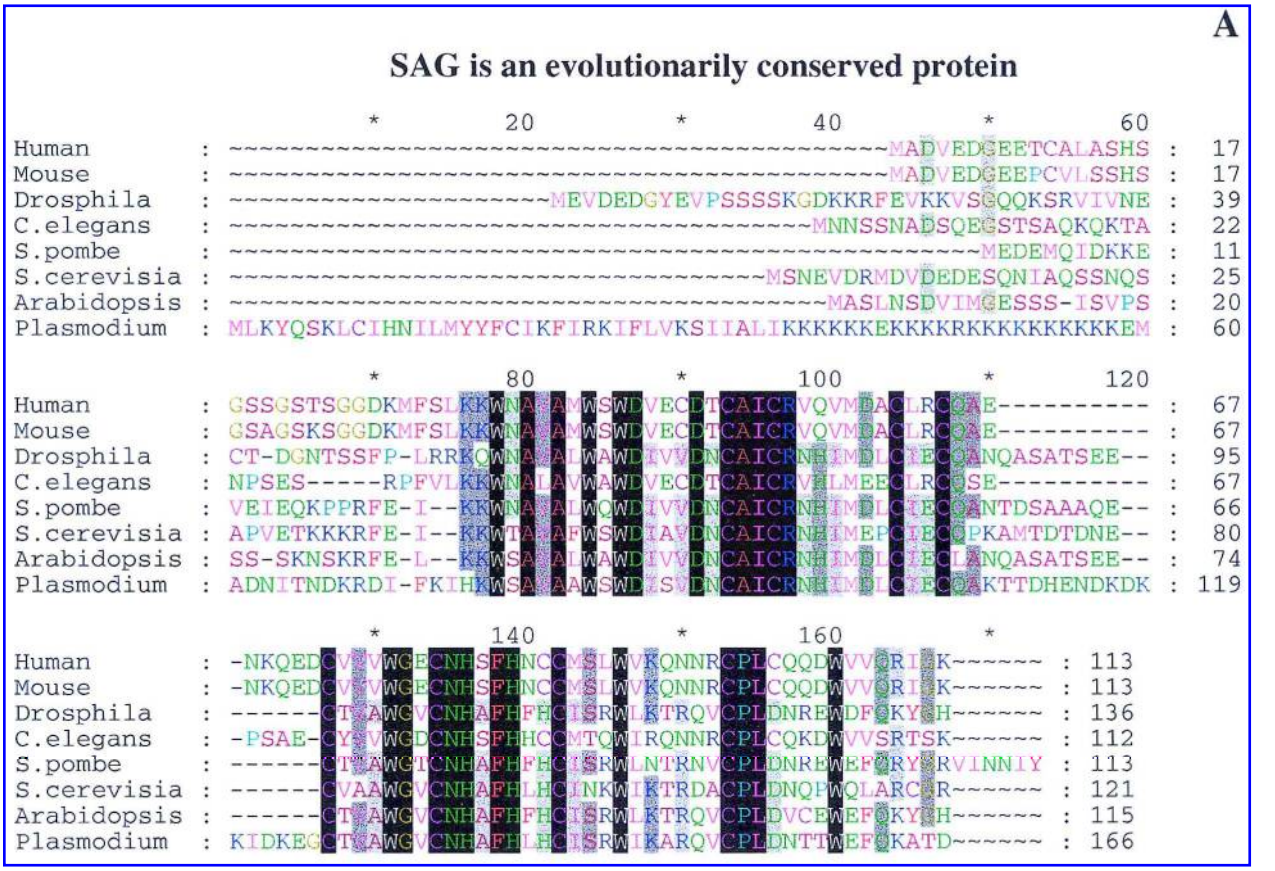
The tissue expression pattern of both SAG family genes is identical in humans. They are ubiquitously distributed with very high expression in heart, skeletal muscle, and testis and less expression in brain, lung, kidney, and placenta (20, 85a). Expression of SAG and ROC1 proteins was also examined in multiple human cancer cell lines originated from carcinomas of prostate (LnCap), bone (U2OS, Saos-2), breast (MDA468, MDA453, MCF7), kidney (293), skin (A431), lung (H1299, H460), cervix (HeLa), colon (HT29), ovary (A90), and brain (SY5Y). As shown in Fig. 3A, SAG and ROC1 are expressed in multiple lines, and the expression level of ROC1 is higher, in general, than that of SAG. The lack of detection of SAG expression in U2OS, A431, MDA453, MCF7, HT29, and H460 is probably due to either insufficient protein loading (see figure legend) or very low expression level. In mouse tissues, SAG is also ubiquitously expressed among many tissues, with the highest level in brain (Fig. 3B). The expression pattern of ROC1 in mouse tissues remains to be established.

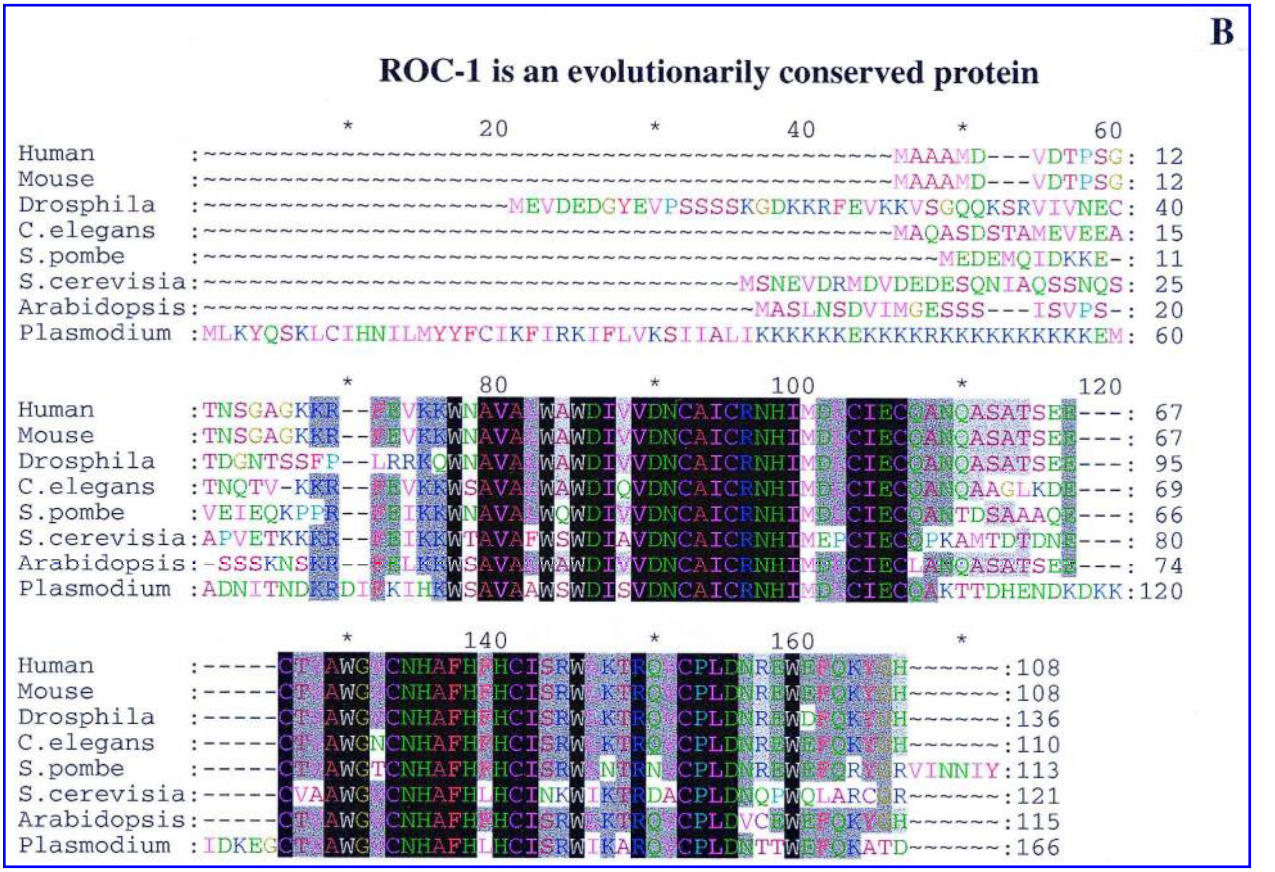
Expression of SAG is also detected in an early stage of mouse development. As shown in Fig. 3C, SAG expression was detected in 9.5–19.5-day-old whole mouse embryos, with a higher level detected in the embryos between days 9.5 and 11.5. These results suggest that SAG play a role in embryonic development. A similar experiment was yet to be performed for ROC1.

Regulation of gene expression

Induction in cultured cells and in mouse brain tissues. As described above, mouse SAG was originally identified by differential display to be an OP-inducible gene in cultured tumor cells. Induction occurs at an early stage upon exposure to OP and lasts for up to 24 h (20). We have extended this *in vitro* observation to an *in vivo* ischemia/reperfusion model in mouse brain. We found that SAG is expressed at a low level in brain tissue and is inducible after middle cerebral artery occlusion with a

FIG. 2. SAG and ROC1 family proteins are evolutionarily conserved. Comparative alignment of SAG (**A**) and ROC1 (**B**) from human, mouse, *Drosophila*, *C. elegans*, *S. pombe*, *S. cerevisiae*, *Arabidopsis*, and *Plasmodium*. Identical residues among all species are blackened, and identical residues in >60% species are shaded.


A
SAG is an evolutionarily conserved protein
Human
Mouse
Drosphila
C.elegans
S.pombe
S.cerevisia
Arabidopsis
Plasmodium


B
ROC-1 is an evolutionarily conserved protein
Human
Mouse
Drosphila
C.elegans
S.pombe
S.cerevisia
Arabidopsis
Plasmodium

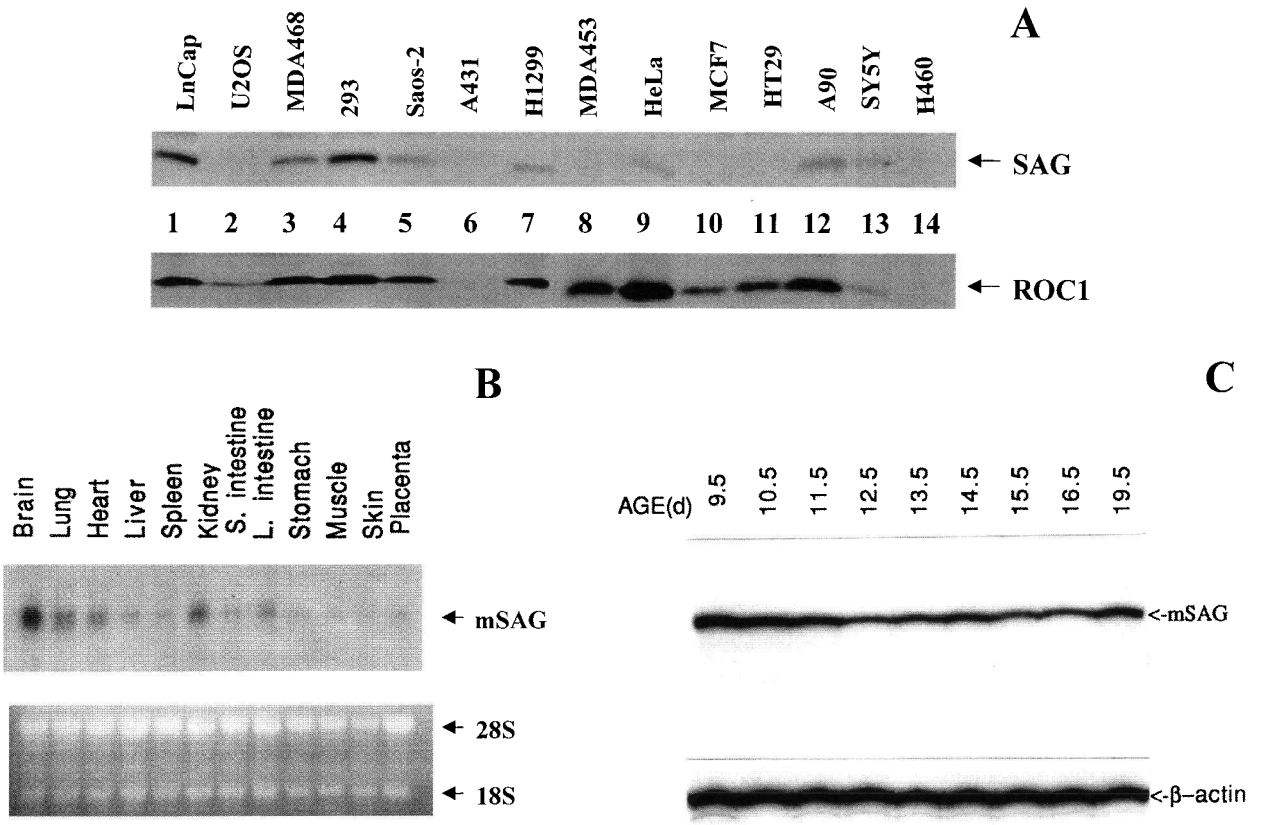


FIG. 3. Expression of SAG and ROC1 in human cancer cell lines and expression of SAG in mouse adult tissues and mouse embryos. (**A**) Cell lysates were prepared from multiple human cancer cell lines and subjected to western blot analysis with anti-SAG and anti-ROC1 antibodies as described (20). The approximate amount of protein loaded is 80 μg in lanes 1, 3–5, 7–9, 12, and 13, 60 μg in lanes 10 and 11, 30 μg in lanes 2 and 14, and 20 μg in lane 6. (**B** and **C**) Total RNA was isolated from mouse adult tissues (B) as well as embryos (C) (provided by Dr. Tom Glaser at the University of Michigan), followed by northern analysis (80) and PCR amplification, respectively. The primers used flank the entire encoding region of mouse SAG: SagTA.01 5′-CGGGATCCCCATGGCCGACGTGAGG-3′ and SagT.02 5′-CGGGATCCTCATTTGCCGATTCTTTG-3′. The PCR was conducted in the presence of ^{35}S-dATP, and products were run in a sequencing gel followed by autoradiography (82).

peak induction at 6–12 h (102). Thus, SAG appears to be a stress response gene. In addition, others have shown that SAG and ROC1 are induced at both RNA and protein levels by mitogenic stimulation (63).

Promoter analysis. We have recently characterized the SAG genomic sequence and found that the SAG gene consists of four exons and three introns (85a). A 4-kb DNA fragment upstream from the translation initiation site of SAG was identified through a computer search (accession no. AC067952.5; nucleotides 31,549–35,538). The promoter sequence analysis reveals that the SAG promoter is enriched with binding sites for many transcription factors, including NF-κB/c-Rel, CREB, E2F, c-myc, c-myb, AP-1, AP-2, Sp-1, STAT, and p53. Further characterization of potential regulation of SAG expression by these transcription factors would lead to a better understanding of SAG expression and provide a molecular basis for its response to environmental stimuli. Genomic structure and the promoter sequence of ROC1 have not been identified.

BIOCHEMICAL PROPERTIES

DNA binding/transcription factor activity

SAG protein belongs to the zinc RING finger protein families by virtue of its $C_3H_2C_3$ motif. Some zinc RING finger proteins have been shown to bind to DNA and act as transcrip-

tional repressors (53, 71), whereas others act as transcriptional activators (13, 65). To examine whether SAG would bind to a specific DNA sequence, we used a technique called CASTing (cyclin amplification and selection of targets), a reiterative selection procedure, described previously for identification of p53 binding sites (30). The target oligonucleotide (75 bp) contains flanking 20-bp PCR primer sites at the 5′-end and 3′-end that bracket a central core of 35-bp random sequence. A complementary strand was made by annealing with the 3′-end 20-bp primer to the target oligonucleotide, followed by primer extension. The double-stranded DNA was then incubated with purified SAG protein and subjected to a gel retardation assay (83). The shifted band was cut out from the gel, PCR-amplified using both the 5′-end and 3′-end primers, and subjected to the next run of gel shift assay. We have performed a total of six rounds of selection. The PCR fragments after the sixth round were cloned by TA cloning vector. More than 50 clones were sequenced, but no consensus sequence was identified (unpublished data). We next examined potential transcriptional regulatory activity of SAG protein using a CAT-reporter assay and found that SAG has neither transactivation nor transrepression activity (84).

RNA binding activity

The zinc RING finger domain of the MDM2 protein has been shown to bind to RNA (22, 46). We next tested whether SAG protein could also bind to RNA. Binding of purified SAG protein to different nucleic acid cellulose conjugates was performed as described (22). As shown in Fig. 4, in contrast to Mdm2, which binds to poly G (22), purified SAG binds to poly U, poly A, and poly C RNA. SAG-poly U binding is very strong and cannot be dissociated with an increasing salt (NaCl) concentration up to 1 *M*. Interestingly, SAG-poly U binding induces SAG oligomerization (also see below). Binding of SAG with poly C and poly A is in a monomeric form, but relatively weak. The binding was dissociated in the presence of 200 m*M* NaCl (data not shown). These results suggest that like Mdm2, SAG is an RNA binding protein. However, the true *in vivo* binding, as well as the biological significance of the binding, remains to be determined.

Protein–protein interaction

ROC1 was first identified in a yeast two-hybrid screening as a cullin-1 binding protein (62). Simultaneously, it was found in a highly purified SCF fraction (87), in a purified VHL complex (42), and in Skp1 and cullin-1/Cdc53 immunoprecipitates (72). Later, it was found that both ROC1 and SAG bind to either one of five cullins (cullin-1 to cullin-5) in both two-hybrid and coimmunoprecipitation assays (63, 85). In addition, SAG was recently found to bind to a regulatory β subunit of casein kinase II in a yeast two-hybrid screening and was phosphorylated by this kinase (74). Finally, we have identified a few positive clones previously unknown to bind to SAG in a yeast two-hybrid screening using the full-length SAG as the bait (unpublished observation). The biological significance of these bindings is being evaluated.

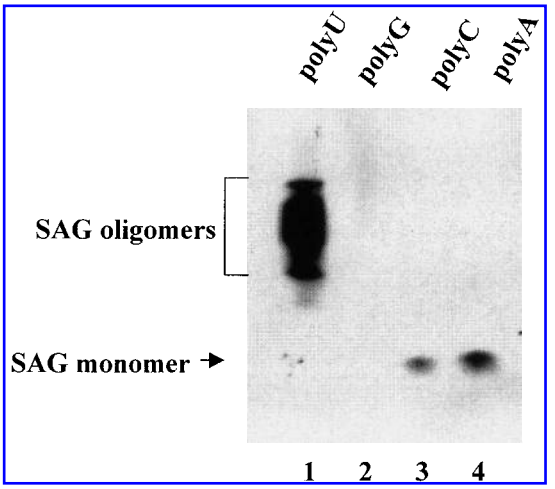


FIG. 4. SAG is an RNA binding protein. SAG protein (0.5–1.0 μg) was incubated at 4°C for 60 min in 300 μl of RNA binding buffer (20 m*M* Tris, pH 7.5, 150 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1% Nonidet P-40, 50 μM $ZnCl_2$, 2% glycerol, and 1 m*M* dithiothreitol) with one of four RNA homopolymer columns (20 μg prewashed; Sigma), conjugated to agarose or cellulose beads. The columns were subsequently washed with 3 ml of RNA binding buffer to remove nonspecifically bound protein from the beads. The beads were then boiled in sodium dodecyl sulfate sample buffer, and the proteins so eluted were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose for western blot analysis using the polyclonal antibody directed against SAG protein as described previously (20).

Ligase activity and modification by ubiquitin-like protein, Rub1/Nedd8 (related to ubiquitin/neural precursor cell-expressed and developmentally down-regulated)

SAG or ROC1 is an activating component of SCF E3 ubiquitin ligase complex as well as VHL E3 ubiquitin ligase complex (51, 90). The ROC1-cullin-1 subunits recruit the Cdc34 ubiquitin conjugating enzyme (E2), whereas Skp1 helps to link the ROC1/cullin-1 subunit and the F-box protein that recognizes the protein target for ubiquitination and degradation. Together, these four subunits make up the minimal SCF ubiquitin ligase complex (19, 90). In the test tube, ROC1 or SAG can bind to cullins to form a "core" ubiquitin ligase that promotes poly-ubiquitination complex in the presence of ubiquitin, E1, and E2 (Cdc34) (97). Mutational analysis revealed that the conserved RING finger domain is required for the ligase activity because ROC1 or SAG mutants with a single or double mutations at $C_3H_2C_3$ residues have no ligase activity even though they still bind to cullin-1 (14, 85). In addition, the ROC1 subunit of SCF and VHL E3 ubiquitin ligase is required for Rub1/Nedd8 modification of cullin-1 and cullin-2 (41) by, at least in the cullin-1 case, facilitating its nuclear accumulation (31). It is now known that Rub1/Nedd8 is conjugated to cullin-1 at Lys^{720} via a covalent linkage, which markedly increases the ability of the ROC1-cullin-1 complex to promote ubiquitin polymerization (96). Disruption of Rub1/Nedd8 modification diminishes IκB ubiquitin ligase activity of ROC1-cullin-1 complex (31). Thus, ROC1 or SAG appears to be a common activating factor of enzyme systems responsible for ubiquitin and Rub1/Nedd8 modification of target proteins.

Metal binding, radical scavenging, and ligase regulation

One feature of the RING finger protein is its ability to bind with zinc to form a "cross-brace" structure (70). Indeed, bacterially expressed and purified SAG protein binds to zinc ion, as well as to copper and iron ions (20, 84). Binding and chelating copper ion by SAG prevents copper ion-induced lipid peroxidation in the test tube (84). SAG also prevent lipid peroxidation induced by AAPH (2,2-azo-*bis*-2-amidinopropane hydrochloride), a free radical generator without metal ion involvement (29). SAG-induced inhibition of lipid peroxidation is completely or partially abolished by pretreatment of SAG with alkylating agents *N*-ethylmaleimide (NEM) or *p*-hydroxymercuric benzoate (PHMB), respectively, suggesting that free SH groups in the SAG molecule are the major contributors to this activity (20). It is noteworthy that the RING finger may not be directly involved in ROS scavenging activity of SAG because (a) metallothionein, a cysteine-rich, RING fingerless protein also has such an activity (20), and (b) some SAG RING finger mutants still retain this activity (84, and unpublished observation). Thus, it is possible that the cysteine residues (which consist of 10.6% of SAG protein), rather than the RING finger motif *per se*, are mainly responsible for observed ROS scavenging activity.

It is of interest to note that PHMB or mercuric chloride, but not NEM, can irreversibly inhibit the ROC1-cullin-1 ubiquitin ligase activity without disrupting the complex (14). Likewise, these reagents also inhibit *in vitro* ubiquitination of IκB by SCF E3 ubiquitin ligase complex (14). The fact that ROC1-cullin-1 ubiquitin ligase activity is insensitive to NEM suggests that sulfhydryl groups in RING cysteine residues are not mediating a thiol-ester ubiquitin transfer probably because they bind to zinc ions and are inaccessible to NEM. However, the critical residues involved in interaction with mercuric ion to induce protein conformation change and inactivation of ROC1-cullin-1 E3 ligase activity remain to be determined (14).

Oligomerization

Bacterially expressed and purified SAG protein is redox-sensitive. Upon exposure to oxidant H_2O_2, SAG forms oligomers as well as monomer doublets due to the formation of the inter- or intramolecular disulfide bonds, respectively. This process can be reversed by the reducing agent dithiothreitol or prevented by pretreatment with NEM (84). In addition, binding with poly U also induces SAG oligomerization (Fig. 4). Although SAG oligomerization

is readily detectable in purified protein as well as in cell lysate prepared from 293 cells transiently transfected with SAG expressing construct (84), it remains to be determined whether such an oligomerization occurs inside cells and, if so, what its biological consequence is.

BIOLOGICAL FUNCTIONS

Apoptosis protection

In vitro *cell culture models.* To study the biological function of SAG, we established stable cell lines that overexpress SAG in two cell models: DLD-1 human colon carcinoma cells and SY5Y human neuroblastoma cells. Compared with the vector-transfected controls, SAG-overexpressed cells are more resistant to apoptosis induced by OP, as well as metal ions (zinc or copper), as measured by morphological observation of the signs of apoptosis, TUNEL assay, and DNA fragmentation. We have also transiently overexpressed SAG in 293 cells and observed a protection against copper-induced apoptosis (20, 77).

In vivo *mouse brain model.* We next extended this *in vitro* observation of SAG protection against apoptosis to an *in vivo* mouse brain model where ischemia/reperfusion-induced brain injury was generated after middle cerebral artery occlusion (MCAO), a process involving ROS production (10, 11, 101). To determine the potential protective role of SAG in this model, recombinant adenovirus expressing wild-type human SAG as well as a SAG mutant, along with the LacZ-expressing control, was injected into mice brain 24 h prior to induction of ischemia. Compared with the LacZ control, infarct volume, a measure of brain damage, was significantly smaller in wild-type SAG-injected mice, but not in SAG mutant-expressing mice. In the areas where SAG is overexpressed, ischemia-induced superoxide production, as well as the number of apoptotic cells, is significantly decreased. Thus, SAG appears to protect ROS-induced brain damage in *in vivo* mice (102).

Requirement of RING domain and ROS scavenging activity. Using two deletion mutants, we have previously shown that the RING domain is required for apoptosis protection (77). To associate activities of metal-ion binding/radical scavenging (RS) and E3 ubiquitin ligase (UL) with apoptosis protection, we have established several stable cell lines in SY5Y cells that express either wild-type SAG (RS+/UL+), ROC1 (RS?/UL+), or two SAG mutants, MM-3 (RS+/UL−), or MM14 (RS−/UL−) (84, 85). Using a cell death ELISA assay, cellular sensitivity to apoptosis induced by zinc ion was determined. As shown in Fig. 5, compared with the vector control (V6), cells expressing wild-type SAG or SAG mutant MM3 are much more resistant, whereas cells expressing SAG mutant MM14 are more sensitive, to zinc-induced cell death. As SAG mutant MM3 with radical scavenging activity (RS+) (84), but without ligase activity (UL−) (85), inhibits apoptosis, it appears that radical scavenging activity rather than E3 ligase activity correlates with the apoptosis-protection phenotype. In addition, like SAG, ROC1 overexpression also protects cells from apoptosis induced by the redox agent.

Mechanism of action. The process of apoptosis can be divided into three distinct phases: initi-

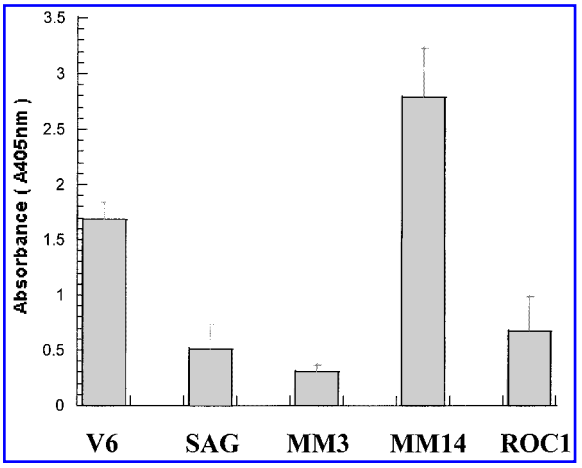


FIG. 5. Overexpression of SAG or ROC1 protects against zinc ion-induced cell death. Human SY5Y neuroblastoma cells were transfected with SAG (SAG), SAG mutant-3 (MM3), mutant-14 (MM14) (84), ROC1 (ROC1), and the empty vector, pcDNA3 (V6). The stable clones were selected, and expression of exogenous protein was detected by western blot analysis (data not shown). The stable transfected cells were then seeded in a 96-well ELISA plate and subjected to zinc sulfate (200 μM) treatment for 16 h. Cell death was measured ($n = 4$) by a cell death detection ELISA kit (Boehringer Mannheim), according to the supplier's instruction. OD value reflects the degree of DNA fragmentation.

ation, effector, and execution/degradation (45). One common apoptosis activation pathway involves release from damaged mitochondria of cytochrome *c*, which forms a complex with Apaf-1 to activate caspase-9, an initiator caspase (49, 68, 106). The caspase-9 in turn activates effector caspases (caspase−3, −6, and −7) that degrade many protein substrates (17), leading to committed apoptosis (49, 89). ROS appear to act mainly at the initiation/activation step of apoptosis because most apoptosis inducers, such as irradiation, UV, and chemicals, produce ROS (38, 88). We therefore hypothesized that SAG-induced apoptosis protection is achieved by its metal ion binding/ROS scavenging activity to protect mitochondria from ROS-induced damage, thus preventing cytochrome *c* release/caspase activation. Indeed, zinc-induced cytochrome *c* release/caspase activation is decreased or delayed in SAG-overexpressed cells (20).

The second potential mechanism by which SAG protects apoptosis is through the ubiquitination and degradation of IκB (62, 87). Degradation of IκB leads to activation of NF-κB, a known apoptosis protector (2, 58, 92, 94). We have explored this possibility by infecting recombinant adenovirus expressing SAG into HeLa cells to see whether it would promote tumor necrosis factor-α (TNF-α)-induced IκB degradation. As shown in Fig. 6, Ad-SAG infection induces a very high expression of SAG protein in HeLa cells that have very low endogenous SAG (not seen in the figure with LacZ infection in a short film exposure time). IκB degradation started to occur 5 min after TNF-α treatment, and a complete degradation was achieved at 20 min after treatment. The IκB level then started to recover and reached a level compatible to that of the untreated control as a result of subsequent NF-κB activition (see Fig. 7), which in turn transactivates IκB expression (2). No significant difference between Ad-SAG and Ad-Lac-Z (used as a control) was observed, except that SAG infection appears to delay the complete recovery of IκB at 60 min. No difference in p65 protein level was found. We next examined the time course of NF-κB activation following IκB degradation by a gel shift assay. As shown in Fig. 7, NF-κB activation started to occur 10 min after TNF-α exposure, immediately following the time course of IκB degradation. Activation reached its maximal level after 20 min up to 60 min in LacZ-infected cells, but to a lesser extent in SAG-infected cells, which correlates with the relatively low level of IκB (see Fig. 6). However, the difference is rather small and considered to be insignificant. Thus, although ROC1/SAG is involved in IκB ubiquitination and degradation in a test tube

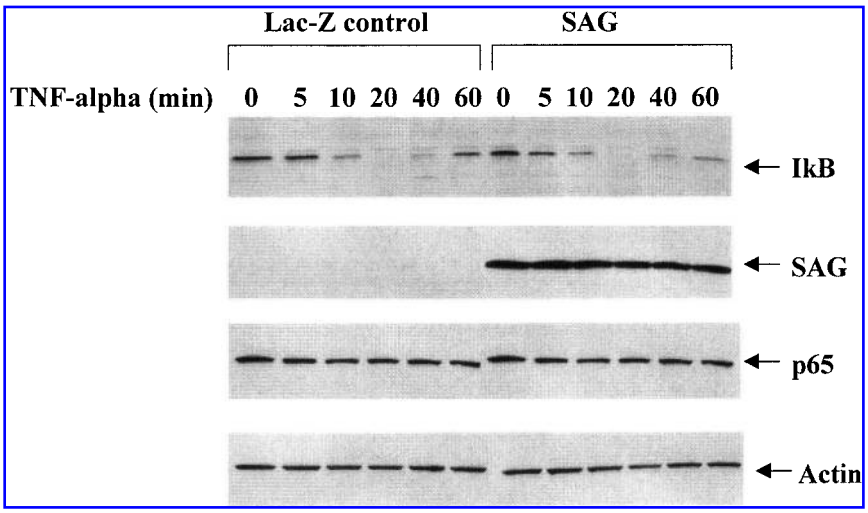


FIG. 6. Overexpression of SAG does not promote IκB degradation. HeLa cells (~7.5×10^5) were seeded in a 100-mm culture dish 18–24 h prior to infection with Ad-SAG and Ad-LacZ control as described (21). Cells were then treated with TNF-α (10 ng/ml) for the indicated periods of time, harvested, lysed, and subjected (60 μg) to western blot analysis (20) with antibodies directed against IκBα, p65, SAG, and β-actin, respectively.

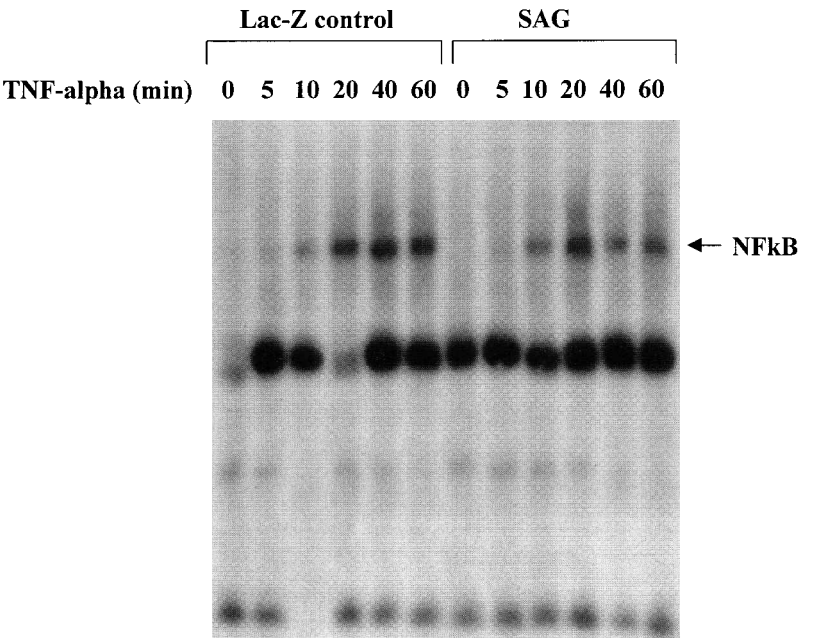


FIG. 7. Overexpression of SAG does not promote NF-κB activation. Nuclear extracts were prepared from Ad-SAG-infected, TNF-α-treated cells (4), and were then subjected (20 μg) to a gel shift assay. The probe used is a 20-bp synthetic oligonucleotide containing the consensus NF-κB binding site: 5′-AGTTGAGGGGACTTTCCCAG-3′. The probe was labeled and assay performed, as described previously (4).

assay (62, 87), SAG appears not to promote IκB degradation/NF-κB activation in this cell model. The results suggest that either SAG is not a rate-limiting factor in this process or the potential role of SAG has been saturated due to a relatively high level of ROC1 in the cells (Fig. 3A).

Growth promotion

Yeast model. To understand SAG function further, we used a yeast genetic approach by taking advantage of the fact that *SAG* is evolutionarily conserved between yeast *Saccharomyces cerevisiae* and human. Targeted disruption of *ySAG*, the yeast homologue of human SAG, induces cell death that is fully rescued by wild-type SAG and ROC1, but not by their ligase-dead mutants, indicating that (a) ySAG is essential for yeast viability, (b) human SAG or ROC1 is its functional homologue, and (c) ligase activity is required (42, 62, 72, 85). Interestingly, cell death induced by *SAG* deletion was accompanied by cell enlargement and abnormal cell-cycle profiling. Chip profiling of the entire yeast genome revealed induction of several G1/S as well as G2/M checkpoint control genes upon SAG withdrawal. Thus, *SAG* appears to control cell-cycle progression in yeast by promoting ubiquitination and degradation of cell-cycle regulatory proteins (85).

Mammalian cell models. We next examined the potential growth-promoting activity of SAG in mammalian cells. Indeed, SAG promotes cell growth under serum-starved conditions. Microinjection of SAG mRNA into quiescent NIH3T3 cells induces S phase entry as determined by [^{3}H]thymidine incorporation. Likewise, overexpression of SAG by either adenovirus infection of immortalized human epidermal keratinocytes (Rhek) or DNA transfection of SY5Y human neuroblastoma cells induces cell proliferation under serum-starved conditions, but not under normal growth conditions (21).

Antisense inhibition of tumor cell growth. SAG appears to promote cell growth. We next examined whether SAG is overexpressed in human tumor tissues and whether it is a dominant oncogene. SAG protein level is elevated in six of 10 human colon carcinoma tissues (60%), as compared with adjacent normal tissues from

the same patient. However, SAG overexpression in preneoplastic cells in the JB6 tumor promotion/progression model did not induce neoplastic transformation, suggesting that SAG is not a dominant oncogene, at least in this model system. Interestingly, when DLD-1 human colon carcinoma cells were transfected with antisense SAG, cell growth both in monolayer and in soft agar was significantly inhibited, suggesting that SAG is important in the maintenance of tumor cell growth (37).

Mechanism of action. As SAG/ROC1 is a component of E3 ubiquitin ligases that promote protein degradation, and as cyclin-dependent kinase inhibitors, including p21, p27, and p57, are degraded through the ubiquitin pathway, we tested whether SAG-induced cell growth is associated with cyclin-dependent kinase inhibitor degradation. No significant difference in the levels of p21 and p57 were found in the vector controls and SAG-overexpressed cells. However, serum starvation-induced accumulation of p27 was significantly reduced by SAG overexpression in two cell models (21). We also correlated the protein levels of SAG and p27 in colon cancer tissues and found an inverse correlation in four of 10 samples (40%). We concluded that SAG-induced growth promotion under serum deprivation is associated at least in part with inhibition of p27 accumulation. In addition, other unknown SAG target protein(s) may also play a role in the process.

CONCLUSIONS AND PERSPECTIVES

In summary, SAG/ROC1 family members are evolutionarily conserved RING finger-containing proteins. They appear to be a key component of E3 ubiquitin ligases to target for degradation of many regulatory proteins (67) that play an important role in the regulation of many key biological processes, including cell-cycle progression, transcription, and signal transduction (43, 56). In addition, SAG has antioxidant activity by binding metal ions and scavenging ROS (84). Biologically, SAG appears to protect against apoptosis induced by redox agents and promote cell growth under stress conditions. Future studies directed to generate somatic knockout cells as well as conditional knockout animals (12, 100) would lead to a better understanding of SAG/ROC1 function and provide a clear answer as to whether they are valid anticancer targets. The gene promoter characterization would help to understand the precise regulation of these proteins at the transcriptional level, whereas elucidation of SAG phosphorylation by casein kinase II (74) and its biological significance would reveal additional regulation at the posttranslational level. Moreover, characterization of proteins that bind to SAG/ROC1 and its biological consequence would further our understanding of their functions and mechanism of action. There is no doubt that the activity of SCF E3 ubiquitin ligases regulates cell growth, oncogenesis, apoptosis, and inflammation by targeting for degradation of p21, p27, β-catenin, IκB, and Wee1, just to name a few (19). As the field progresses and we have a better understanding of the specificity of these ligases in targeting a particular protein or pathway, SCF E3 ubiquitin ligases could eventually become the promising targets for cancer and inflammation drug discovery.

ABBREVIATIONS

Nedd8, neural precursor cell-expressed and developmentally down-regulated; NEM, *N*-ethylmaleimide; NF-κB, nuclear factor-κB; OP, 1,10-phenanthroline; PHMB, *p*-hydroxymercuric benzoate; Rbx, RING box protein; RING, really interesting new gene; ROC, regulator of cullins; ROS, reactive oxygen species; Rub1, related to ubiquitin; SAG, sensitive to apoptosis gene; SCF, Skp/cullin/F-box; TNF-α, tumor necrosis factor-α; VHL, von Hippel–Lindau.

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