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Thiol-linked Peroxidase Activity of Human Sensitive to Apoptosis Gene (SAG) Protein

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SAG (sensitive to apoptosis gene), a novel zinc RING finger protein, which is redox responsive and protects mammalian cells from apoptosis, is a metal chelator and a potential reactive oxygen species (ROS) scavenger, but its antioxidant properties have not been completely defined. Here, we show that SAG possesses a potent peroxidase property to decompose hydrogen peroxide in the presence of dithiothreitol (DTT). However, without DTT as a reducing equivalent, SAG was not able to destroy hydrogen peroxide. The peroxidase activity was completely abolished by the reaction of SAG with N-ethylmaleimide (NEM), a chemical modification agent for the sulfhydryl of proteins. These observations suggested that the sulfhydryl of cysteines in SAG could function as strong nucleophiles to destroy hydrogen peroxide. In addition to the peroxidase activity used to remove hydrogen peroxide, SAG also showed t-butylhydroperoxide (t-BOOH) and fatty acid hydroperoxide-selective peroxidase activity.

Keywords: SAG; Peroxidase; Sulfhydryl groups; Hydrogen peroxide

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion O_2^-, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) are generated *in vivo* from the incomplete reaction of oxygen during aerobic metabolism or stimulated host phagocytes, or from exposure to environmental agents such as radiation and redox cycling agents.[1–3] These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modifications and strand breaks.[4,5] Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyze the dismutation of O_2^- to H_2O_2 and O_2,[6] catalase, and peroxidases, which remove hydrogen peroxide and hydroperoxides.[7–9]

SAG (sensitive to apoptosis gene) is a novel, evolutionally conserved, zinc RING finger protein that protects cells from apoptosis induced by redox reagents.[10] In human tissues, SAG is ubiquitously expressed at high levels in skeletal muscles, heart, and testis. SAG is localized in both the cytoplasm and the nucleus of cells. SAG encodes a protein that consists of 113 amino acids including 12 cysteine residues with a molecular weight of 12.6 kDa.[10] Several biological roles of SAG have been suggested.[10–12] Besides being involved in the protection of cells from apoptosis induced by oxidative stress, other biological functions have been considered. For instance, it has been proposed that on account of its high cysteine content, SAG might play an antioxidant role in the cell through a metal chelator or ROS scavenger.[10,11] It has also been reported that SAG interacts with and is phosphorylated by casein kinase 2,[12] although the biological meaning of this interaction remains to be elucidated.

In the present study, in addition to the several already known functions of SAG, we report that SAG exhibits thiol-dependent peroxidase activity.

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MATERIALS AND METHODS

Materials

Hydrogen peroxide (30% solution), xylenol orange, dithiothreitol (DTT), N-ethylmaleimide (NEM), bovine catalase, ampicillin, isopropyl β-D-thiogalactoside (IPTG), t-butylhydroperoxide (t-BOOH), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical (St. Louis, MO, USA). Bio-Rad protein assay kit and electrophoresis reagents were obtained from Bio-Rad (Hercules, CA, USA). Ni–NTA–agarose resin was obtained from Qiagen (Valencia, CA, USA). Linoleic acid hydroperoxide (LAOOH) was prepared by incubating 0.1 mM linoleic acid with 10 μg ml^{-1} soybean lipoxygenase in 50 mM Hepes–NaOH (pH 7.0) at room temperature for 10 min.[13] The concentration of LAOOH was determined spectrophotometrically ($\varepsilon_{234} = 25{,}000\ M^{-1}\,cm^{-1}$).

Purification of SAG

For SAG purification, *Escherichia coli* BL21 (DE3) harboring pET14b-SAG were grown in Luria–Bertani broth supplemented with ampicillin at 37°C, and expression of His-tagged SAG was induced by 1 mM IPTG for 4 h. Bacteria were harvested and washed twice with Buffer A (20 mM Tris–HCl, pH 8.0, 200 mM NaCl, and 0.2 mM PMSF). After the cells were suspended and lysed in Buffer B (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 8.0), the cellular debris was removed by centrifugation at 30,000 g for 10 min at 4°C. The supernatant was then subjected to affinity chromatography through Ni–NTA–agarose resin which was equilibrated with Buffer B. After washing the resin with Buffer C (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 5.9), His-tagged SAG was eluted with Buffer D (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 4.5). The elution of His-tagged SAG was monitored by staining with Coomassie blue following electrophoresis on 15% SDS–polyacrylamide gel.[14] The SAG fractions were pooled and dialyzed against Buffer E (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 0.5 mM PMSF). Protein concentrations were measured according to the method of Bradford using bovine serum albumin as a standard.

Determination of Peroxidase Activity of SAG

To determine the peroxidase activity of SAG, the reaction was started by the addition of 250 μM H_2O_2 into the 10 μl reaction mixture containing various concentrations of DTT, 40 mM Hepes buffer at pH 7.0, and an indicated amount of SAG protein, and then incubated at 37°C. The measurement of the remaining H_2O_2 was performed by the method as described.[15] Hydrogen peroxide oxidizes ferrous (Fe^{2+}) to ferric ion (Fe^{3+}) selectively in dilute acid and the resulting ferric ions can be determined using a ferric sensitive dye, xylenol orange, as an indirect measure of hydrogen peroxide concentration. Reaction mixtures were added 990 μl FOX solution (0.1 mM xylenol orange, 0.25 mM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H_2SO_4) and incubated at room temperature for 30 min, and absorbance was measured at 560 nm.

Chemical Modification of SAG With NEM

The modification of cysteine in SAG (100 μg ml^{-1}) with 1–20 mM NEM was carried out as previously described.[16]

Replicates

The data represent average values with standard deviations obtained from three independent experiments.

RESULTS

To study the peroxidase activity of SAG, we have expressed human SAG in *E. coli* as a fusion protein using the pET14b expression vector.[12] The fusion protein contains a hexahistidine sequence attached to the amino-terminus of SAG protein. As shown in Fig. 1, SAG protein was purified to near homogeneity by the Ni–NTA affinity chromatography.

When DTT was absent in the reaction mixture, human SAG hardly catalyzed the decomposition of H_2O_2. However, SAG has a capacity to remove H_2O_2 in a time-dependent manner in the presence

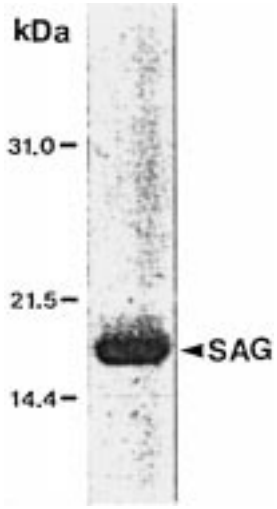

FIGURE 1 His-tagged SAG purified by Ni–NTA (Qiagen) chromatography was subjected to electrophoresis on a 15% (w/v) SDS–polyacrylamide gel and stained with Coomassie blue.

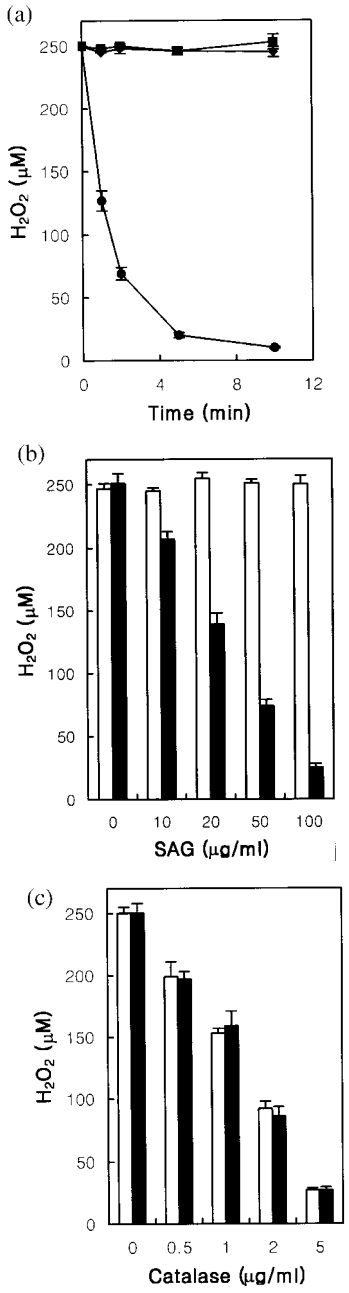

FIGURE 2 Removal of H_2O_2 by SAG or catalase. (A) Time-dependent removal of H_2O_2 by SAG. Peroxidase reaction was carried out in a 10 μl reaction mixture containing 40 mM Hepes (pH 7.0), 10 mM DTT alone (■), 100 μg ml^{-1} SAG alone (◆), or both 10 mM DTT and 100 μg ml^{-1} SAG (●). At indicated times, the concentration of the remaining H_2O_2 was measured with the use of xylenol orange as described.[15] (B) Dose-dependent removal of H_2O_2 by SAG. Different amounts of SAG protein were incubated with 250 μM H_2O_2 in the absence (white) and presence (black) of 10 mM DTT at 37°C for 5 min, and the concentration of the remaining H_2O_2 was assayed. (C) Dose-dependent removal of H_2O_2 by catalase. Different amounts of catalase were incubated with 250 μM in the absence (white) and presence (black) of 10 mM DTT at 37°C for 5 min, and the concentration of remaining H_2O_2 was assayed.

of 10 mM DTT (Fig. 2A). DTT itself did not significantly destroy H_2O_2. Within 5 min, 100 μg ml^{-1} SAG can destroy about 90% of 250 μM H_2O_2 in the presence of DTT. Figure 2B shows the H_2O_2-destroying profile as a function of the concentration of SAG. SAG required DTT to remove H_2O_2, but catalase eliminated H_2O_2 regardless of the existence of DTT (Fig. 2C), indicating that DTT with sulfhydryl groups did not react with H_2O_2. The Linweaver–Burk plot constructed from the initial H_2O_2 removal rates in a reaction mixture containing 10 mM DTT, 100 μg ml^{-1} SAG, 40 mM Hepes buffer at pH 7.0, and various concentrations of H_2O_2 showed that the K_m value of SAG for H_2O_2 was 0.23 mM. Because SAG is a cysteine-rich protein, we reasoned that the DTT-dependent peroxidase activity is possibly due to the sulfhydryl groups. It can be suggested that an inactive form of SAG was converted to the active form by DTT, presumably back to the thiol form. The peroxidase activity of SAG was dependent on the concentration of DTT (Fig. 3). It is noteworthy that although 10 mM DTT did not show significant H_2O_2-removing activity, 8 μM of SAG (calculated from 100 μg ml^{-1} protein, based on the molecular weight of 12,600 Da for human SAG), removed 90% of H_2O_2 within 5 min at 37°C. This result indicates the strong nucleophilicity of cysteine residues in SAG.

To examine the possibility that cysteines in SAG might act as catalytic groups, SAG was reacted with the cysteine-specific alkylating reagent NEM. Figure 4 shows that the H_2O_2-removal activity of SAG decreased as a function of the concentration of NEM. The loss of peroxidase activity by NEM and the effect of DTT on the peroxidase activity indicate that free sulfhydryl groups in the SAG molecule are the major contributors to this activity.

We investigated other kinds of peroxides as substrates for SAG. In addition to the peroxidase activity used to remove hydrogen peroxide, SAG also showed t-BOOH and fatty acid hydroperoxide-selective peroxidase activity (Fig. 5).

DISCUSSION

SAG is a cysteine-rich, metal-binding protein that is found in a wide range of organisms from yeast to human.[10] SAG is evolutionally conserved, with a 55 or 70% identity, respectively, between human and yeast or human and *C. elegans* sequences. Although SAG is thought to serve as an important line of defense against oxidative stress-induced damage and redox-induced apoptosis, the biological roles of SAG have not been completely elucidated. SAG could be a growth-essential gene by functioning as an antioxidant molecule to protect cells against ROS-induced death or by promoting cell growth through other mechanisms.[10] Because of its peculiar structure, characterized by a large content of thiol groups,

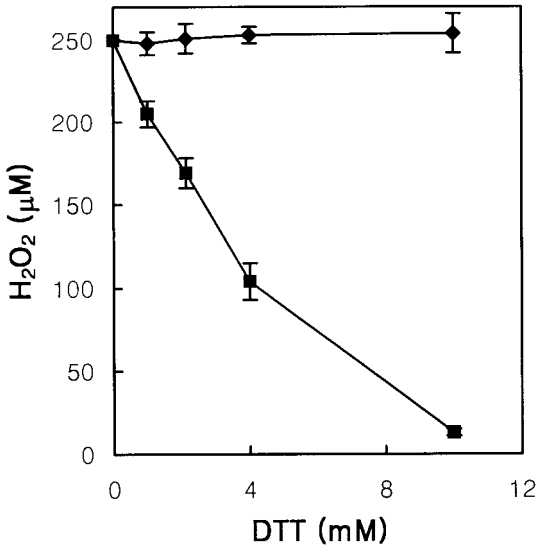

FIGURE 3 The concentration-dependent effect of DTT on the peroxidase activity of SAG. The reaction mixture contained 250 μM H_2O_2, various concentrations of DTT, along with 100 μg ml^{-1} SAG (■) or without SAG (◆). After incubation at 37°C for 10 min, the concentration of remaining H_2O_2 was measured.

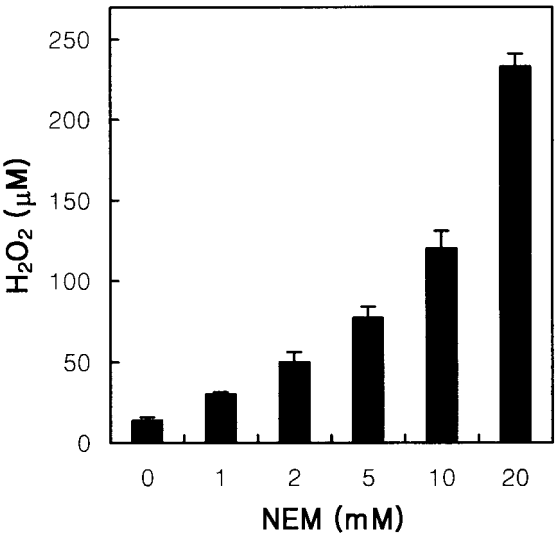

FIGURE 4 The concentration-dependent effect of NEM on the peroxidase activity of SAG. The reaction mixture contained 250 μM H_2O_2, 10 mM DTT, various concentrations of NEM, and 100 μg ml^{-1} SAG. After incubation at 37°C for 10 min, the concentration of remaining H_2O_2 was measured.

SAG is endowed with strong antioxidant and nucleophilic properties. Based on the prevention of LDL oxidation by copper ion and 2,2-azo-bis-2-amidinopropane hydrochloride (AAPH), a free radical generator, it has been suggested that SAG plays a role as a metal, especially Cu, chelator or free radical scavenger through sulfhydryl groups.[10,11] SAG contains neither heme nor a flavin prosthetic group and it does not possess any activity of known antioxidant enzymes including catalase, SOD, and glutathione peroxidase. The present study indicates that SAG is a thiol peroxidase, in which the sulfhydryls of cysteines could function as strong nucleophiles to destroy peroxides including H_2O_2, t-BOOH, and fatty acid hydroperoxide. It can be assumed that cysteine residues of SAG may be easily oxidized due to their strong nucleophilicity. The oxidized cysteine residues can be converted to the active sulfhydryl groups by reaction with excess thiol such as DTT in vitro. When 10 mM DTT was replaced with 10 mM GSH, SAG no longer elicited peroxidase activity. We proposed that the NADPH-dependent thioredoxin system is one of the candidates that can be used as a physiological reducing equivalent, although this proposal remains to be proven.

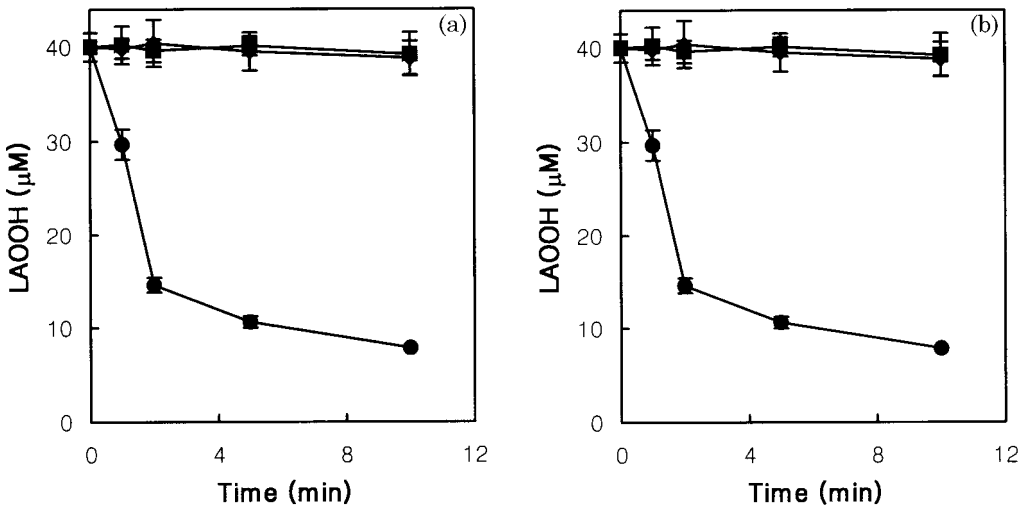

FIGURE 5 The thiol peroxidase activity of SAG with (A) t-BOOH or (B) LAOOH acts as a substrate. Peroxidase reaction was carried out in a 10 μl reaction mixture containing 40 mM Hepes (pH 7.0), 10 mM DTT alone (■), 100 μg ml^{-1} SAG alone (◆), or both 10 mM DTT and 100 μg ml^{-1} SAG (●), and 100 μM t-BOOH or 40 mM LAOOH as a substrate. At indicated times, the concentration of remaining peroxide was measured with the use of xylenol orange as described.[15]

Analogies with cysteine in SAG showing a strong nucleophilicity were reported by other laboratories. Thioredoxin peroxidase (TPx) is a recently discovered 25 kDa peroxidase that reduces H_2O_2 with the use of electrons provided by either NADPH-dependent thioredoxin system or by thiols, like DTT.[8,9] A database search revealed a number of proteins from a variety of organisms that are similar to TPx. These homologous proteins were named the peroxiredoxin (Prx) family.[17,18] This protein family is widespread in nature and its high degree of conservation suggests a biological importance of this type of enzymes. In addition to TPx, this family includes, the AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase,[17] a mitochondrial protein induced during the differentiation of Friend erythroleukemia cells[19] which is also known as SP22,[20] two proteins showing a natural killer cell-enhancing action *in vitro* and named NKEF-A and B,[21,22] DirA, a major iron-repressible polypeptide, of *Corynebacterium diphtheriae*,[23] a 28 kDa secretory protein from rat olfactory epithelium,[24] a 29 kDa protein of *Entamoeba histolytica*,[25] and tryparedoxin peroxidase of the *Crithidia fasciculate* peroxidase system.[26] This family of proteins shares amino acid sequence homology, and the catalytic activity depends on one or two specific cysteine(s).

Another type of thiol-linked peroxidases exists which shares a similar catalytic activity to that of peroxiredoxin, however, the amino acid sequence of these proteins are completely different from Prx. This family includes human serum albumin[27] and human ceruloplasmin,[28] and it is not known that any specific cysteine or nonspecific cysteine are involved in the thiol-dependent peroxidase activity of these proteins. SAG may belong to this family of thiol peroxidases. It remains to be studied if whether or not 12 cysteines of SAG are involved in the protection against oxidative stress as a non-specific way or if any specific cysteines are involved in the catalysis. In conclusion, the localization (cytoplasm and nucleus),[10] the inducible nature under oxidative stress,[10] and the K_m value indicate that SAG is, presumably, involved in the protection of cells against oxidative stress. The *in vivo* function of SAG remains to be explored further, but the SAG warrants further investigation as a new type of antioxidant enzyme.

Acknowledgements

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