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Human Golgi Antiapoptotic Protein Modulates Intracellular Calcium Fluxes

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Golgi antiapoptotic protein (GAAP) is a novel regulator of cell death that is highly conserved in eukaryotes and present in some poxviruses, but its molecular mechanism is unknown. Given that alterations in intracellular Ca$^{2+}$ homeostasis play an important role in determining cell sensitivity to apoptosis, we investigated if GAAP affected Ca$^{2+}$ signaling. Overexpression of human (h)-GAAP suppressed staurosporine-induced, capacitative Ca$^{2+}$ influx from the extracellular space. In addition, it reduced histamine-induced Ca$^{2+}$ release from intracellular stores through inositol trisphosphate receptors. h-GAAP not only decreased the magnitude of the histamine-induced Ca$^{2+}$ fluxes from stores to cytosol and mitochondrial matrices, but it also reduced the induction and frequency of oscillatory changes in cytosolic Ca$^{2+}$. Overexpression of h-GAAP lowered the Ca$^{2+}$ content of the intracellular stores and decreased the efficacy of IP$_3$-induced Ca$^{2+}$ release from intracellular stores through inositol trisphosphate receptors. h-GAAP not only decreased the magnitude of the histamine-induced Ca$^{2+}$ fluxes from stores to cytosol and mitochondrial matrices, but it also reduced the induction and frequency of oscillatory changes in cytosolic Ca$^{2+}$. Overexpression of h-GAAP lowered the Ca$^{2+}$ content of the intracellular stores and decreased the efficacy of IP$_3$-induced Ca$^{2+}$ release from intracellular stores. Opposite effects were obtained when h-GAAP was knocked down by siRNA. Thus, our data demonstrate that h-GAAP modulates intracellular Ca$^{2+}$ fluxes induced by both physiological and apoptotic stimuli.

INTRODUCTION

Recently, a novel regulator of cell death was identified (Gubser et al., 2007). This protein was named Golgi antiapoptotic protein (GAAP) because of its predominant localization in the Golgi and its ability to suppress apoptosis. GAAP is a predicted seven-transmembrane protein and was identified initially in certain poxviruses (vaccinia virus and camelpox virus) where it affects virus virulence. GAAPs are highly conserved in a broad range of organisms including human, orangutan, dog, mouse, rat, Xenopus laevis, and zebrafish, and related proteins are present in Drosophila and Arabidopsis. Human (h)-GAAP is expressed ubiquitously in human tissue and shares 73% aa identity with viral (v)-GAAP. Stable expression of either v-GAAP or h-GAAP suppressed cell death induced by a broad variety of intrinsic and extrinsic apoptotic stimuli. Conversely, knockdown of h-GAAP in tissue culture cells by siRNA resulted in cell death.

Ca$^{2+}$ functions as a ubiquitous intracellular signal to many different biological processes. Ca$^{2+}$-induced signaling arises from Ca$^{2+}$ entry across the plasma membrane and/or release from intracellular stores, predominantly the endoplasmic reticulum (ER) and Golgi. Ca$^{2+}$ is released from intracellular stores by inositol-1,4,5-trisphosphate (IP$_3$), which interacts with IP$_3$ receptors (IP$_3$R) that are Ca$^{2+}$ release channels present in the ER and Golgi (Pinton et al., 1998). Furthermore, IP$_3$R activity is modulated by Ca$^{2+}$ itself, ATP, phosphorylation, and interacting proteins (Foskett et al., 2007). Ca$^{2+}$ that enters the cytosol activates cytosolic enzymes and is taken up by mitochondria, which play an important role in decoding Ca$^{2+}$ signals during normal cell physiology (Berridge et al., 2003). Mitochondrial Ca$^{2+}$ uptake is mediated by a low-affinity Ca$^{2+}$ uniporter that senses the high Ca$^{2+}$ microdomains that are established at the tight junctions between the ER and mitochondria (Rizzuto et al., 1998). Recently, tight junctions with a putative role in Ca$^{2+}$ signaling were also observed between Golgi and mitochondria (Dolman et al., 2005). Alterations in the finely tuned intracellular Ca$^{2+}$ homeostasis and compartmentalization contribute to the induction of apoptosis. The switch from the control of physiological functions to the involvement in this death program most likely entails changes in the tightly regulated spatio-temporal Ca$^{2+}$ signaling pattern affecting cytosolic effector proteins and effector organelles (Orrenius et al., 2003). Ca$^{2+}$

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Abbreviations used: ATP, adenosine triphosphate; ER, endoplasmic reticulum; GAAP, Golgi antiapoptotic protein; HA, hemagglutinin; IP$_3$, inositol-1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; PMCA, plasma membrane Ca$^{2+}$-ATPase; SERCA, sarcoendoplasmic reticulum Ca$^{2+}$-ATPase; siRNA, small interfering RNA; TG, thapsigargin.
signaling between storage organelles and mitochondria plays an important role in sensitizing cells to apoptosis (Pintor and Rizzuto, 2006). Molecular and pharmacological approaches that lowered Ca2+ levels in the stores and thereby reduced Ca2+ signaling to the mitochondria, protected cells from apoptosis, whereas conditions that increased Ca2+ levels in the stores had the opposite effect (Ma et al., 1999; Nakamura et al., 2000; Pintor et al., 2001; Pintor and Rizzuto, 2006). Moreover, both antiapoptotic (e.g., Bcl-2 and Bcl-XL) and proapoptotic (e.g., Bax and Bak) partially localize at the ER to regulate Ca2+ signaling (Oakes et al., 2003; Chen et al., 2004; White et al., 2005).

On the basis of the localization of h-GAAP at intracellular Ca2+ stores and the established importance of intracellular Ca2+ signaling in sensitizing cells to apoptosis induction, we hypothesized that the antiapoptotic role of h-GAAP may be mediated by modulating the Ca2+ content of these stores and/or the flux of Ca2+ between these stores and the closely opposed mitochondria. Here, evidence is presented that h-GAAP alters intracellular Ca2+ fluxes induced by both a physiological stimulus (histamine) and an apoptotic stimulus (staurosporine).

**MATERIALS AND METHODS**

**Cells and Medium**

U2OS-neo and U2OS-h-GAAP cell lines were described previously (Gubser et al., 2007). Cells were grown in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum and 10 μg/ml Ciprofix (Bayer, Newbury, Berks, United Kingdom) at 37°C in a 5% CO2 atmosphere.

**Antibodies, Conjugates, and Reagents**

Mouse monoclonal antibodies against IP3R3, calnexin, Bcl-2, and pxallin were obtained from BD Transduction Laboratories (Lexington, KY); Mouse mAb against protein disulfide isomerase (PDI) was from StressGen (San Diego, CA), against Bcl-XL from Santa Cruz Biotechnology (Santa Cruz, CA) and against t-tubulin from Upstate Laboratories (Lake Placid, NY). Rabbit polyclonal antibodies against Bax and Bak were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody (Rbt 476) against IP3R (all isoforms) was described previously (Ma et al., 2002). Coelenterazine-W, coelenterazine-X, fura-2 acetoxyethyl ester (fura-2/AM), and Rhod-2/AM were from Molecular Probes (Eugene, OR), histamine and iomycin from Sigma-Aldrich (Poole, Dorset, United Kingdom), 2-APB from Calbiochem (La Jolla, CA), and STS from Roche (East Sussex, United Kingdom).

**RNA Interference**

Sequences of small interfering RNA1 (siRNA1) and siRNA2 (Ambion, Austin, TX) were described previously (Gubser et al., 2007). Cells were grown to 50% confluency in six-well plates and transfected with 1 μg of each of the above siRNAs using siFECTamine (IC-Vecl; www.ic vec.com) according to the manufacturer’s instructions.

**Immunoprecipitation**

Coimmunoprecipitation was performed as described for Bcl-2 interaction with IP3R (Chen et al., 2004). Abs used were anti-IP3R Ab (BD Biosciences, Poole, United Kingdom; 1:200), anti-HA mAb (1:200) and the control Ab used was a mouse IgG2a Ab-1 (Stratch Scientific, Berkshire, United Kingdom; 1:150). Proteins were resolved by SDS-PAGE and transferred onto Hybond-P PVDF membranes (Amersham, Bucks, United Kingdom).

**Digital Imaging Microscopy of Cytosolic and Mitochondrial Ca2+ Concentrations**

Cells (3 × 10^4) seeded on 24-mm glass coverslips were coloaded with 3 μM fura-2/AM and 5 μM rhod-2/AM for 25 min at 37°C and used for monitoring simultaneous changes in mitochondrial and cytosolic Ca2+ concentration as described (Visch et al., 2004). The fura-2 and rhod-2 dyes were excited at 380 and 540 nm, respectively. The fura-2 fluorescence emission ratio at 492 nm was monitored as a measure of the free cytosolic Ca2+ concentration after alternating excitation at 340 and 380 nm. In all experiments, the fluorescence emission signal was normalized to its prestimulatory value, which was set at 1.

**RESULTS**

**h-GAAP Decreases Cytosolic and Mitochondrial Ca2+ Rises Triggered by an Apoptotic Stimulus**

In this study, possible effects of h-GAAP on intracellular Ca2+ signaling were investigated using U2OS cells that stably expressed hemagglutinin (HA)-tagged h-GAAP predominantly at the Golgi but also at the ER (hereafter referred to as h-GAAP cells; Gubser et al., 2007). Essentially the same results were obtained with two independently constructed h-GAAP cell lines. Except in experiments in which parental U2OS cells were transfected with siRNA, U2OS cells containing the empty plasmid vector were used as a control (hereafter referred to as U2OS-neo cells). Western blot analysis was performed to exclude the possibility that h-GAAP overexpression affected the expression levels of ER chaperones or Bcl-2 family members. The data show that the expression levels of calnexin, PDI, Bcl-2, Bcl-XL, Bak, and Bak in h-GAAP cells was not altered compared with U2OS-neo cells (Supplementary Figure S1).

Previously, staurosporine (STS)-induced apoptosis was demonstrated to be partially, but significantly, inhibited in h-GAAP cells (Gubser et al., 2007). The exact mechanism by which STS induces cell death is unknown, but STS-induced cell death is at least partially Ca2+-dependent (Oakes et al., 2003; Chen et al., 2004; White et al., 2005). Therefore, we first addressed a possible role of h-GAAP in the STS-induced changes in intracellular Ca2+ homeostasis (Boehning et al., 2003). To this end, cells were coloaded with the cytosolic Ca2+ indicator fura-2 and the mitochondrial Ca2+ indicator rhod-2, treated with STS, and analyzed by digital imaging microscopy. Initial measurements of the resting cytosolic Ca2+ concentration using fura-2 revealed no detectable differences between U2OS-neo and h-GAAP cells (resting fura-2 ratio in U2OS-neo cells was 0.35 ± 0.02, n = 85 cells, measured on 5 d; resting fura-2 ratio in h-GAAP was 0.35 ± 0.01, n = 85 cells, measured on 5 d). On addition of 2 μM STS, U2OS-neo cells displayed a gradual increase in both mitochondrial and cytosolic Ca2+ concentration (Figure 1, A and B). Both increases were virtually abolished by 2-aminoethoxydiphenylborate (2-APB), a drug that suppresses extracellular Ca2+ entry by inhibiting store-operated Ca2+ channels and indicating that STS acts to stimulate the capac-

**Luminescence Monitoring of Ca2+**

For luminescence measurement of Ca2+, 5 × 10^4 cells were seeded on 13-mm glass coverslips, transfected with targeted aequorin (Pintor et al., 1998) using FuGENE 6 reagent (Roche), and analyzed as described (Visch et al., 2004; Visch et al., 2006).

**Ca2+ Fluxes**

Ca2+ fluxes were performed as described (Kasri et al., 2006). Briefly, cells were grown to confluency, permeabilized with saponin, loaded with 4Ca2+, and washed with efflux medium containing 4 μM thapsigargin (TG) to block ATP-dependent Ca2+ uptake. IP3-stimulated Ca2+ release was initiated by the addition of efflux medium containing the indicated concentration of IP3, and 2 min later the efflux medium was collected and counted for radioactivity. After correction for the passive leak induced by TG alone, the amount of radioactivity released by IP3 was expressed as a percentage of the total amount of radioactivity present in the stores as determined by addition of the Ca2+ ionophore A23187. To assess the rate of passive Ca2+ leakage induced by TG alone, the efflux medium was replaced every 2 min during 18 min. At the end of the experiment, all residual radioactivity was released by incubation with 1 ml of 2% SDS for 30 min. For each data point, the amount of radioactivity that was still present in the stores was calculated, expressed as a percentage of the total amount present at the onset of the experiment, and plotted as a function of time.

**Calculations**

Data are presented as mean values ± SEM. Differences were tested for significance using the Student’s t test.
f-GAAP binds to IP3R, decreases the STS-induced increase in both mitochondrial and cytosolic Ca2+ concentration. This effect is shown in Figure 1C, where h-GAAP cells showed a much larger STS-induced increase in mitochondrial and cytosolic Ca2+ concentration than untreated cells. Moreover, the upstroke of the Ca2+ transient was virtually completely inhibited by 2-APB. These results show that h-GAAP reduces the STS-induced increase in mitochondrial and cytosolic Ca2+ concentration, which depends on the influx of Ca2+ across the plasma membrane.

h-GAAP Decreases Histamine-induced Rises in Cytosolic and Mitochondrial Ca2+

To gain more insight into a possible role of h-GAAP in the regulation of intracellular Ca2+ signaling, we investigated its effects on the increase in cytosolic and mitochondrial Ca2+ concentration evoked by the IP3-generating hormone histamine. To prevent capacitative Ca2+ entry, stimulation with histamine was performed in the absence of extracellular Ca2+, i.e., after dye-loading, cells were washed in Ca2+-free medium, transferred to Ca2+-free medium containing 0.5 mM EGTA, and stimulated with histamine 1 min later. Under these conditions, 100 μM histamine evoked a transient rise in cytosolic Ca2+ concentration, the amplitude of which was significantly reduced in h-GAAP cells (Figure 1C). Moreover, the upstroke of the Ca2+ transient induced by histamine appeared slower in h-GAAP cells than in U2OS-neo cells. To test the effects of h-GAAP on histamine-induced mitochondrial Ca2+ uptake, cells were transfected with a mitochondrial-targeted variant of the Ca2+-sensitive photoprotein aequorin. Resting Ca2+ levels in the mitochondrial matrix were not significantly altered in h-GAAP cells (U2OS-neo cells, 0.038 ± 0.010 μM, n = 10 coverslips, measured on 3 d; h-GAAP cells, 0.034 ± 0.009 μM, n = 14 coverslips, measured on 3 d). Histamine evoked a transient increase in mitochondrial Ca2+ concentration, the amplitude of which was significantly decreased in h-GAAP cells (p < 0.05; Figure 1D). Western blotting using an antibody that recognizes all three IP3R isoforms revealed no detectable differences in the amount of IP3R in U2OS-neo and h-GAAP cells (Figure 1E). These results indicate that h-GAAP can decrease the histamine-induced increase in mitochondrial and cytosolic Ca2+ concentration, mediated by IP3Rs and, in this case, depending solely on the release of Ca2+ from intracellular stores.

h-GAAP Coimmunoprecipitates with the IP3R

The above results suggested that h-GAAP might interact with IP3Rs thus providing a potential explanation for its inhibitory effect on the flux of Ca2+ through these receptors. Because currently there exists no good antibody against h-GAAP for immunoprecipitation purposes, we made use of a different approach: we made use of a mitochondrial-targeted variant of the Ca2+-sensitive photoprotein aequorin. Resting Ca2+ levels in the mitochondrial matrix were not significantly altered in h-GAAP cells (U2OS-neo cells, 0.038 ± 0.010 μM, n = 10 coverslips, measured on 3 d; h-GAAP cells, 0.034 ± 0.009 μM, n = 14 coverslips, measured on 3 d). Histamine evoked a transient increase in mitochondrial Ca2+ concentration, the amplitude of which was significantly decreased in h-GAAP cells (p < 0.05; Figure 1D). Western blotting using an antibody that recognizes all three IP3R isoforms revealed no detectable differences in the amount of IP3R in U2OS-neo and h-GAAP cells (Figure 1E). These results indicate that h-GAAP can decrease the histamine-induced increase in mitochondrial and cytosolic Ca2+ concentration, mediated by IP3Rs and, in this case, depending solely on the release of Ca2+ from intracellular stores.

h-GAAP Coimmunoprecipitates with the IP3R
the HA-tag that was fused to h-GAAP. The results obtained show that the anti-HA mAb coprecipitated IP_3R subtype 3 (IP_3R3; Figure 2A), which is the most abundant subtype in U2OS cells (data not shown). The specificity of this reaction was confirmed by the failure of the antibody to precipitate any sarcoendoplasmic reticulum Ca^{2+}/H^{+}-ATPase isoenzyme 2b (SERCA2b), the predominant SERCA protein in non-muscle cells. In the reciprocal experiment, immunoprecipitation of IP_3R3 brought down h-GAAP (Figure 2B). These results demonstrate that h-GAAP interacts, either directly or indirectly, with the IP_3R.

**h-GAAP Alters the IP_3-induced Ca^{2+} Release Response from Intracellular Ca^{2+} Stores in Permeabilized Cells**

Because h-GAAP can be coprecipitated with IP_3Rs, we next assessed the possibility that it might affect the characteristics of the IP_3-induced Ca^{2+} release response. To gain access to the IP_3Rs, we made use of a permeabilized cell system. Cells were permeabilized with saponin, loaded to steady state with ^{45}Ca^{2+}, washed to remove excess ATP using an efflux medium containing the SERCA inhibitor TG to prevent Ca^{2+}-reuptake, and challenged with either the Ca^{2+} ionophore A23187, to determine total releasable ^{45}Ca^{2+} or the indicated concentration of IP_3. After 2 min, the time required for completion of the rapid phase of the IP_3-induced Ca^{2+} release response, the medium was removed, and the amount of ^{45}Ca^{2+} released was determined, corrected for passive ^{45}Ca^{2+} leakage, and expressed as percentage of total releasable ^{45}Ca^{2+}. Maximum stimulation with IP_3 released a significantly smaller fraction of total releasable Ca^{2+} in h-GAAP cells (p < 0.005, Figure 2C). The latter finding was not likely to be due to a decrease in the number of IP_3Rs because immunoblot analysis of cell lysates revealed no detectable difference in expression of the most abundant subtype 3 (Figure 2A).

Calculation of the EC_{50} value showed that h-GAAP did not affect the sensitivity for IP_3 (1.35 ± 0.33 and 1.28 ± 0.14 μM IP_3 for U2OS-neo and h-GAAP cells, respectively). We observed a small effect of h-GAAP on the cooperativity of the IP_3-induced Ca^{2+} release response, as measured by calculation of the Hill coefficient (1.22 ± 0.22 and 1.02 ± 0.15 for U2OS-neo and h-GAAP cells, respectively), but this difference was not statistically significant. These results indicate that h-GAAP decreases the efficacy of IP_3 without altering its potency. It remains to be established whether this involves a direct or indirect interaction between h-GAAP and IP_3Rs.

**h-GAAP Lowers the Amount of Stored Ca^{2+} in Intact Cells**

Next, we measured the steady-state Ca^{2+} content of IP_3R-regulated Ca^{2+} stores in intact cells expressing h-GAAP. To this end, cells were loaded with the cytosolic Ca^{2+} indicator fura-2, transferred to a Ca^{2+} free medium, and treated either with the Ca^{2+} ionophore ionomycin (1 μM) or the SERCA inhibitor BHQ (20 μM). Because the Ca^{2+} content of these stores is maintained by a pump-leak system, SERCA inhibi-
tion will lead to passive release of stored Ca\(^{2+}\) into the cytosol. The results show that both BHQ (Figure 3A) and ionomycin (Figure 3B) evoked a transient rise in cytosolic Ca\(^{2+}\) concentration, the amplitude of which was significantly reduced in h-GAAP cells. Importantly, and in contrast to what was observed with histamine, the upstroke of the Ca\(^{2+}\) transients induced by BHQ and ionomycin was unaltered in h-GAAP cells.

To investigate the effects of h-GAAP on the Ca\(^{2+}\) concentration in ER and Golgi separately, cells were transfected with organelle-targeted aequorins, permeabilized with saponin at 20 h after transfection, and assayed for ATP-dependent Ca\(^{2+}\) uptake under "cytosolic" conditions at a free Ca\(^{2+}\) concentration of 0.1 \(\mu M\). In both organelles the steady-state Ca\(^{2+}\) concentration appeared lower (~20%) in h-GAAP cells (p < 0.01, Figure 3, C and D). Importantly, no major differences in the initial rate of Ca\(^{2+}\) uptake were observed, indicating that h-GAAP does not alter the SERCA pump capacity of the intracellular stores.

To establish more firmly that h-GAAP has an effect on the steady-state Ca\(^{2+}\) content of the intracellular stores, we next determined this content at different times after transfection of parental U2OS cells with h-GAAP siRNA. Comparison with parental U2OS cells transfected with control siRNA revealed no detectable difference at 20 h after transfection (Figure 3E). At 40 h after transfection, however, the amplitude of the ionomycin-induced increase in cytosolic Ca\(^{2+}\) concentration was significantly higher (~35%) in h-GAAP down-regulated cells (p < 0.001). Organelle-targeted aequorins revealed that this increase in Ca\(^{2+}\) concentration occurred in both ER and Golgi (p < 0.05, Figure 3F).

**hGAAP Decreases the Sensitivity to Histamine Induction of Oscillatory Cytosolic Ca\(^{2+}\) Changes**

In the experiments described thus far, cells were stimulated with a "pharmacological" concentration of histamine (100 \(\mu M\)). In the remainder of this study, we assessed the possible consequences of these findings on Ca\(^{2+}\) signaling in intact cells under more "physiological" conditions. Cells were loaded with fura-2 and superfused with medium containing (sub)micromolar concentrations of histamine. Digital imaging microscopy of individual cells revealed that 0.3 and 1.0 \(\mu M\) histamine increased the cytosolic Ca\(^{2+}\) concentration in ~70 and ~95% of the U2OS-neo cells, respectively. For h-GAAP cells, these values were ~20 and ~70%, respectively, indicating a reduced sensitivity to hormonal induction of an increase in cytosolic Ca\(^{2+}\) concentration. In a small percentage of the responding U2OS-neo cells (~5–10% at 1.0 \(\mu M\) histamine), the initial large Ca\(^{2+}\) transient was followed by Ca\(^{2+}\) oscillations (Figure 4, A and B). These Ca\(^{2+}\) oscil-
DISCUSSION

In this study, evidence is presented that h-GAAP, a novel regulator of cell death, reduces both extracellular Ca²⁺ influx evoked by staurosporine, a widely used apoptosis inducer, and intracellular Ca²⁺ release evoked by histamine, known to exert its effect on intracellular Ca²⁺ through IP3.

Using a permeabilized cell system, which allows experimental control of the cytosolic compartment, h-GAAP overexpression was demonstrated to lower the efficacy of IP₃, as demonstrated by a reduction of the maximum amount of (A23187-) releasable Ca²⁺ that could be released by IP₃. Because neither the amount of IP₃Rs nor affinity for IP₃ were detectably altered in h-GAAP-overexpressing cells, this result suggests that h-GAAP either decreases the IP₃-sensitive part of the (A23187-) releasable Ca²⁺ store or, alternatively, decreases the Ca²⁺ release properties of the IP₃-channels. In intact cells, h-GAAP overexpression was shown to reduce the amount of total (ionomycin- or BHQ-) releasable Ca²⁺, consistent with a reduced filling state of the intracellular stores. Consistent with the above results, the cytosolic and mitochondrial Ca²⁺ increases in response to a pharmacological histamine concentration (100 μM) were down-regulated in intact cells overexpressing h-GAAP and up-regulated when h-GAAP was knocked down. Furthermore, stimulation with a more close to physiological concentration of histamine (1 μM) revealed that h-GAAP rendered cells less sensitive to the induction of cytosolic Ca²⁺ oscillations, characteristic for these low concentrations of histamine. Together, these data suggest that h-GAAP reduces both the total amount of releasable Ca²⁺ and its maximum amount that can be released by IP₃, thereby attenuating IP₃-induced cytosolic and mitochondrial Ca²⁺ signaling.

How h-GAAP exerts these effects remains to be established. In this study, we showed that h-GAAP inhibits the influx of extracellular Ca²⁺ influx and decreases the IP₃-mediated release of Ca²⁺ from the stores. In addition, we showed that h-GAAP coprecipitated with IP₃Rs, suggesting an interaction. Such an interaction, which may be either direct or indirect, could be involved in the ability of h-GAAP to attenuate IP₃-induced cytosolic and mitochondrial Ca²⁺ signaling.
to suppress Ca$^{2+}$ fluxes. However, the observed effects of h-GAAP may equally well be explained by its ability to reduce the filling state of the Ca$^{2+}$ stores. Therefore, the importance of this interaction for the observed function of h-GAAP requires further investigation.

A reduction in Ca$^{2+}$ filling state of the intracellular stores is usually associated with an increase in capacitative Ca$^{2+}$ entry across the plasma membrane, resulting in an increase in cytosolic Ca$^{2+}$ concentration. However, under resting conditions no such increase in cytosolic Ca$^{2+}$ concentration was observed in cells overexpressing h-GAAP, despite a decrease in the amount of total releasable Ca$^{2+}$. This result suggests that h-GAAP exerts an inhibitory effect on the process of capacitative Ca$^{2+}$ entry. In agreement with this idea, the STS-induced increase in cytosolic and mitochondrial Ca$^{2+}$ concentration, which depended completely on the presence of extracellular Ca$^{2+}$ and was abolished by 2-APB, an inhibitor of capacitative Ca$^{2+}$ entry channels, was decreased in cells overexpressing h-GAAP and was increased in cells in which this protein was down-regulated. STS and histamine increased the cytosolic Ca$^{2+}$ concentration with different kinetics, a relatively slow increase after addition of STS versus a relatively fast increase after stimulation with histamine. In contrast to STS, histamine readily increased the cytosolic Ca$^{2+}$ concentration in the absence of extracellular Ca$^{2+}$, reflecting the IP$_3$-induced release of Ca$^{2+}$ from intracellular stores. The present finding that h-GAAP lowers the histamine-induced increase in cytosolic Ca$^{2+}$ concentration in the absence of external Ca$^{2+}$ strongly supports the idea that GAAP exerts its effect by reducing the IP$_3$-mediated release of Ca$^{2+}$ from intracellular stores. In doing so, h-GAAP likely also reduces the capacitative entry of Ca$^{2+}$. It remains to be established whether a similar mechanism underlies the inhibitory effect of h-GAAP on the STS-induced entry of extracellular Ca$^{2+}$.

The ability of h-GAAP to interfere with intracellular Ca$^{2+}$ signaling provides a plausible explanation for its ability to suppress apoptosis. This idea is supported by observations that modulation of IP$_3$R activity (by antisense knockdown, genetic deletion, or using a cell-permeable inhibitory peptide that interferes with the IP$_3$R-cytochrome c interaction) rendered cells less sensitive to apoptosis triggered by both intrinsic and extrinsic pathways (Joseph and Hajnoczky, 2007). Moreover, Bcl-2 and Bcl-X$_L$ two major antiapoptotic proteins, interact with the IP$_3$R and alter its activity, though in opposite ways: Bcl-2 decreases the IP$_3$R opening probability, whereas Bcl-X$_L$ increases it (Oakes et al., 2003; Chen et al., 2004; White et al., 2005). The mechanism by which alterations in cellular Ca$^{2+}$ handling sensitize or protect cells from apoptosis are as yet incompletely understood. Decreases in the amount of Ca$^{2+}$ available for signaling may prevent cytoxic Ca$^{2+}$ fluxes between the stores and the cytosol and/or the mitochondria. Cell death-regulating proteins have also been linked to cellular metabolism (Hammerman et al., 2004; Kim et al., 2005; Skulachev, 2006). The ability of Bcl-X$_L$ to increase the IP$_3$R opening probability was shown to elevate Ca$^{2+}$ oscillations, resulting in enhanced mitochondrial activity and cellular bioenergetics under steady-state conditions (White et al., 2005). h-GAAP may suppress apoptosis by down-regulating cytosolic and/or mitochondrial Ca$^{2+}$ rises. Exactly how h-GAAP modulates intracellular Ca$^{2+}$ signaling and suppresses apoptosis remains to be elucidated. The observation that h-GAAP is present in an immunoprecipitable complex with the IP$_3$R and modulates IP$_3$-induced Ca$^{2+}$ signaling does not necessarily imply that it acts directly on the IP$_3$R. h-GAAP may be part of a larger IP$_3$R complex comprising also Bcl-2 and/or Bcl-XL and may exert its effects on Ca$^{2+}$ homeostasis through this complex.

In conclusion, h-GAAP is a novel protein that modulates both capacitative Ca$^{2+}$ entry and IP$_3$-mediated Ca$^{2+}$ release. Altogether, these data show that h-GAAP has an important role in the cross-talk between the intracellular Ca$^{2+}$ stores, the cytosol and the mitochondria, and this may explain how h-GAAP plays a decisive role in regulating cell death by apoptosis.

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