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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 Ca2+ homeostasis play an important role in determining cell sensitivity to apoptosis, we investigated if GAAP affected Ca2+ signaling. Overexpression of human (h)-GAAP suppressed staurosporine-induced, capacitative Ca2+ influx from the extracellular space. In addition, it reduced histamine-induced Ca2+ release from intracellular stores through inositol trisphosphate receptors. h-GAAP not only decreased the magnitude of the histamine-induced Ca2+ fluxes from stores to cytosol and mitochondrial matrices, but it also reduced the induction and frequency of oscillatory changes in cytosolic Ca2+. Overexpression of h-GAAP lowered the Ca2+ content of the intracellular stores and decreased the efficacy of IP3, providing possible explanations for the observed results. Opposite effects were obtained when h-GAAP was knocked down by siRNA. Thus, our data demonstrate that h-GAAP modulates intracellular Ca2+ 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. Ca2+ functions as a ubiquitous intracellular signal to many different biological processes. Ca2+-induced signaling arises from Ca2+ entry across the plasma membrane and/or release from intracellular stores, predominantly the endoplasmic reticulum (ER) and Golgi. Ca2+ is released from intracellular stores by inositol-1,4,5-trisphosphate (IP3), which interacts with IP3 receptors (IP3Rs) that are Ca2+ release channels present in the ER and Golgi (Pinton et al., 1998). Furthermore, IP3R activity is modulated by Ca2+ itself, ATP, phosphorylation, and interacting proteins (Foskett et al., 2007). Ca2+ that enters the cytosol activates cytosolic enzymes and is taken up by mitochondria, which play an important role in decoding Ca2+ signals during normal cell physiology (Berridge et al., 2003). Mitochondrial Ca2+ up-take is mediated by a low-affinity Ca2+ uniporter that senses the high Ca2+ 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 Ca2+ signaling were also observed between Golgi and mitochondria (Dolman et al., 2005). Alterations in the finely tuned intracellular Ca2+ 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 Ca2+ signaling pattern affecting cytosolic effector proteins and effector organelles (Orrenius et al., 2003). Ca2+...
signaling between storage organelles and mitochondria plays an important role in sensitizing cells to apoptosis (Pinton and Rizzuto, 2006). Molecular and pharmacological approaches that lowered Ca\(^{2+}\) levels in the stores and thereby reduced Ca\(^{2+}\) signaling to the mitochondria, protected cells from apoptosis, whereas conditions that increased Ca\(^{2+}\) levels in the stores had the opposite effect (Ma et al., 1999; Nakamura et al., 2000; Pinton et al., 2001; Pinton and Rizzuto, 2006). Moreover, both antiapoptotic (e.g., Bcl-2 and Bcl-X\(_L\)) and proapoptotic (e.g., Bak and Bak) partially localize at the ER to regulate Ca\(^{2+}\) signaling (Oakes et al., 2003; Chen et al., 2004; White et al., 2005).

On the basis of the localization of h-GAAP at intracellular Ca\(^{2+}\) stores and the established importance of intracellular Ca\(^{2+}\) signaling in sensitizing cells to apoptosis induction, we hypothesized that the antiapoptotic role of h-GAAP may be mediated by modulating the Ca\(^{2+}\) content of these stores and/or the flux of Ca\(^{2+}\) between these stores and the closely opposed mitochondria. Here, evidence is presented that h-GAAP alters intracellular Ca\(^{2+}\) 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 \(\mu\)g/ml Ciproxin (Bayer, Newbury, Berks, United Kingdom) at 37°C in a 5% CO\(_2\) atmosphere.

**Antibodies, Conjugates, and Reagents**

Mouse monoclonal antibodies against IP\(_3\), R3, calnexin, Bcl-2, and paxillin 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 b- tubulin III 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-N, fur-2 acetoxymethyl ester (fura-2/AM), and Rhod-2/AM were from Molecular Probes (Eugene, OR); histamine and iodomycin 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 \(\mu\)g of each of the above siRNAs using siFECTamine (IC-Vec; www.icvec.com) according to the manufacturer’s instructions.

**Immunoprecipitation**

Coimmunoprecipitation was performed as described for Bcl-2 interaction with IP\(_3\) (Chen et al., 2004). Abs used were anti-IP\(_3\), R3 Ab (BD Biosciences, Poole, United Kingdom; 1:200), anti-HA mAb (1:200) and the control Ab used was a mouse IgG2A Ab-1 (Strate Scientific, Bedfordshire, 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 Ca\(^{2+}\) Concentrations**

Cells (3 \(\times\) 10\(^4\)) seeded on 24-mm glass coverslips were loaded with 3 \(\mu\)M fura-2/AM and 5 \(\mu\)M rhod-2/AM for 25 min at 37°C and used for monitoring simultaneous changes in mitochondrial and cytosolic Ca\(^{2+}\) 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 Ca\(^{2+}\) 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.

**Luminescence Monitoring of Ca\(^{2+}\)**

For luminescence measurement of Ca\(^{2+}\), 5 \(\times\) 10\(^5\) cells were seeded on 13-mm glass coverslips, transfected with targeted aequorin (Pinton et al., 1998) using FuGENE 6 reagent (Roche), and analyzed as described (Visch et al., 2004; Visch et al., 2006).

**\(45\)Ca\(^{2+}\) Fluxes**

\(45\)Ca\(^{2+}\) fluxes were performed as described (Kasri et al., 2006). Briefly, cells were grown to confluence, permeabilized with saponin, loaded with \(45\)Ca\(^{2+}\), and washed with efflux medium containing 4 \(\mu\)M thapsigargin (TG) to block ATP-dependent Ca\(^{2+}\) uptake. IP\(_3\)-stimulated Ca\(^{2+}\) release was initiated by the addition of efflux medium containing the indicated concentration of IP\(_3\), 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 IP\(_3\) was expressed as a percentage of the total amount of radioactivity present in the stores as determined by addition of the Ca\(^{2+}\) ionophore A23187. To assess the rate of passive Ca\(^{2+}\) 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 \(\pm\) SEM. Differences were tested for significance using the Student’s \(t\) test.

**RESULTS**

**h-GAAP Decreases Cytosolic and Mitochondrial Ca\(^{2+}\) Rises Triggered by an Apoptotic Stimulus**

In this study, possible effects of h-GAAP on intracellular Ca\(^{2+}\) 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-X\(_L\), Bax, 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 Ca\(^{2+}\)-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 Ca\(^{2+}\) homeostasis (Boehning et al., 2003). To this end, cells were coloaded with the cytosolic Ca\(^{2+}\) indicator fura-2 and the mitochondrial Ca\(^{2+}\) indicator rhod-2, treated with STS, and analyzed by digital imaging microscopy. Initial measurements of the resting cytosolic Ca\(^{2+}\) 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 \(\pm\) 0.02, \(n = 85\) cells, measured on 5 d; resting fura-2 ratio in h-GAAP was 0.35 \(\pm\) 0.01, \(n = 85\) cells, measured on 5 d). On addition of 2 \(\mu\)M STS, U2OS-neo cells displayed a gradual increase in both mitochondrial and cytosolic Ca\(^{2+}\) concentration (Figure 1, A and B). Both increases were virtually abolished by 2-aminooethoxy-diphenylborate (2-APB), a drug that suppresses extracellular Ca\(^{2+}\) entry by inhibiting store-operated Ca\(^{2+}\) channels and indicating that STS acts to stimulate the capac-
its effects on the increase in cytosolic and mitochondrial gradient SDS-PAGE.

F. de Mattia et al. and Mitochondrial Ca2+ h-GAAP Decreases Histamine-induced Rises in Cytosolic plasma membrane. STS-induced Ca2+ regulation of intracellular Ca2+ medium (data not shown). Importantly, expression of h-GAAP expression, respectively (Gubser shown before to decrease h-GAAP or be without effect on siRNA) or siRNA2 (hereafter referred to as control siRNA), were tested for their response to STS at 2 d after transfection, at which time they were shown before to be still alive (Gubser et al., 2007). Cells transfected with h-GAAP siRNA showed a much larger STS-induced increase in mitochondrial and cytosolic Ca2+ concentration than untreated U2OS-neo cells (Figures 1, A and B) or parental U2OS cells treated with either control siRNA or transfection reagent alone (data not shown). Also in h-GAAP siRNA-treated cells, the STS-induced increase in mitochondrial and cytosolic Ca2+ concentration 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 IP3R antibody that recognizes all three subtypes 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 reduce 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 and, in this case, 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...
the HA-tag that was fused to h-GAAP. The results obtained show that the anti-HA mAb coprecipitated IP$_3$R subtype 3 (IP3R3; 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 IP3R3 brought down h-GAAP (Figure 2B). These results demonstrate that h-GAAP interacts, either directly or indirectly, with the IP$_3$R.

**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$_3$Rs, 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$_3$R, 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$_3$Rs 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$_3$Rs.

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

Next, we measured the steady-state Ca$^{2+}$ content of IP$_3$R-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-
h-GAAP reduces the Ca$^{2+}$ filling state of the intracellular stores. (A and B) Fura-2-loaded cells were treated either with 20 μM BHQ (A) or 1 μM ionomycin (B) and changes in cytosolic Ca$^{2+}$ were monitored as described in Figure 2C. Right panels, the average ± SEM of the peak amplitude (A) or integrated curve (B) from three independent experiments performed in duplicate. * p < 0.05. (C and D) Cells transfected with ER (C) and Golgi-targeted (D) aequorins were permeabilized with saponin, and then their Ca$^{2+}$ uptake and content were determined by active loading of the stores in a perfusion medium containing ATP and 0.1 μM free Ca$^{2+}$. Depicted are typical traces (left) and average ± SEM values of three or four measurements (right). ** p < 0.01. (E) Ca$^{2+}$ content of the intracellular stores (average ± SEM) as analyzed by digital imaging microscopy at 20 and 40 h after transfection of the indicated siRNAs. The average Ca$^{2+}$ content of siRNA control-transfected cells was set at 100%. *** p < 0.001. (F) Ca$^{2+}$ filling state of the ER (left) and Golgi (right) in control siRNA and h-GAAP siRNA transfected cells as determined with targeted aequorins at 40 h after transfection. Average values ± SEM of four coverslips is shown. One of three representative experiments is shown. * p < 0.05.

**hGAA Decreases the Sensitivity to Histamine Induction of Oscillatory Cytosolic Ca$^{2+}$ Changes**

In the experiments described thus far, cells were stimulated with "pharmacological" concentration of histamine (100 μM). 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 μM 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 μM histamine), the initial large Ca$^{2+}$ transient was followed by Ca$^{2+}$ oscillations (Figure 4, A and B). These Ca$^{2+}$ oscil-
Figure 4. h-GAAP reduces the sensitivity to hormonal induction of cytosolic Ca\(^{2+}\) oscillations and reduces their frequency. (A and B) Fura-2-loaded cells were stimulated with different concentrations of histamine at the indicated time and monitored for their Ca\(^{2+}\) response. (A) Average percentage of the responding cells (±SEM) that produce Ca\(^{2+}\) oscillations. ** p < 0.01. (B) Representative traces of three oscillating neo and three h-GAAP cells. (C and D) Fura-2-loaded cells transfected with either control siRNA or h-GAAP siRNA were stimulated with different concentrations of histamine at the indicated time and monitored for their Ca\(^{2+}\) response. (C) Representative histamine-induced Ca\(^{2+}\) oscillations of three cells transfected with either siRNA. (D) Average percentage of cells (±SEM) that respond with Ca\(^{2+}\) oscillations to the indicated histamine concentrations. * p < 0.05.

DISCUSSION

In this study, evidence is presented that h-GAAP, a novel regulator of cell death, reduces both extracellular Ca\(^{2+}\) influx evoked by staurosporine, a widely used apoptosis inducer, and intracellular Ca\(^{2+}\) release evoked by histamine, known to exert its effect on intracellular Ca\(^{2+}\) through IP\(_{3}\).

Using a permeabilized cell system, which allows experimental control of the cytosolic compartment, h-GAAP over-expression was demonstrated to lower the efficacy of IP\(_{3}\), as demonstrated by a reduction of the maximum amount of total (A23187-) releasable Ca\(^{2+}\) that could be released by IP\(_{3}\). Because neither the amount of IP\(_{3}\)R nor their affinity for IP\(_{3}\) were detectably altered in h-GAAP–overexpressing cells, this result suggests that h-GAAP either decreases the IP\(_{3}\)-sensitive part of the (A23187-) releasable Ca\(^{2+}\) store or, alternatively, decreases the Ca\(^{2+}\) release properties of the IP\(_{3}\)-channels. In intact cells, h-GAAP overexpression was shown to reduce the amount of total (ionomycin- or BHQ-) releasable Ca\(^{2+}\), consistent with a reduced filling state of the intracellular stores. Consistent with the above results, the cytosolic and mitochondrial Ca\(^{2+}\) increases in response to a pharmacological histamine concentration (100 \(\mu\)M) 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 \(\mu\)M) revealed that h-GAAP rendered cells less sensitive to the induction of cytosolic Ca\(^{2+}\) oscillations, characteristic for these low concentrations of histamine. Together, these data suggest that h-GAAP reduces both the total amount of releasable Ca\(^{2+}\) and its maximum amount that can be released by IP\(_{3}\), thereby attenuating IP\(_{3}\)-induced cytosolic and mitochondrial Ca\(^{2+}\) signaling.

How h-GAAP exerts these effects remains to be established. In this study, we showed that h-GAAP inhibits the influx of extracellular Ca\(^{2+}\) influx and decreases the IP\(_{3}\)-mediated release of Ca\(^{2+}\) from the stores. In addition, we showed that h-GAAP coprecipitated with IP\(_{3}\)R, suggesting an interaction. Such an interaction, which may be either direct or indirect, could be involved in the ability of h-GAAP
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 added to the presence of extracellular Ca\(^{2+}\), 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.

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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**REFERENCES**


dent and slowly reversible action on store-operated calcium entry channels.

Cell Calcium 34, 97–108.


Pinton, P., Pozzan, T., and Rizzuto, R. (1998). The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) store, with functional properties distinct from those of the endoplasmic reticulum. EMBO J. 17, 5298–5308.


