Molecular Biology of the Cell
Vol. 20, 3638–3645, August 15, 2009

Human Golgi Antiapoptotic Protein Modulates Intracellular Calcium Fluxes

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Submitted May 11, 2009; Accepted June 16, 2009
Monitoring Editor: John York

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, 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 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_3Rs) that are Ca^{2+} release channels present in the ER and Golgi (Pinton *et al.*, 1998). Furthermore, IP_3R 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 spatiotemporal Ca^{2+} signaling pattern affecting cytosolic effector proteins and effector organelles (Orrenius *et al.*, 2003). Ca^{2+}

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–05–0385) on June 24, 2009.

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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_3R, 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 (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., Bax 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 μg/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_3R3, 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 α-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-N, fura-2 acetoxymethyl ester (fura-2/AM), and Rhod-2/AM were from Molecular Probes (Eugene, OR), histamine and ionomycin 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-Vec; www.icvec.com) according to the manufacturer's instructions.

Immunoprecipitation

Coimmunoprecipitation was performed as described for Bcl-2 interaction with IP_3R (Chen *et al.*, 2004). Abs used were anti-IP_3R3 Ab (BD Biosciences, Poole, United Kingdom; 1:200), anti-HA mAb (1:200) and the control Ab used was a mouse IgG2a Ab-1 (Stratech 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 (3×10^5) 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 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+}, 5×10^4 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 confluency, permeabilized with saponin, loaded with $^{45}Ca^{2+}$, and washed with efflux medium containing 4 μM 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 ± 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 ± 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 Ca^{2+} concentration (Figure 1, A and B). Both increases were virtually abolished by 2-aminoethoxy-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-

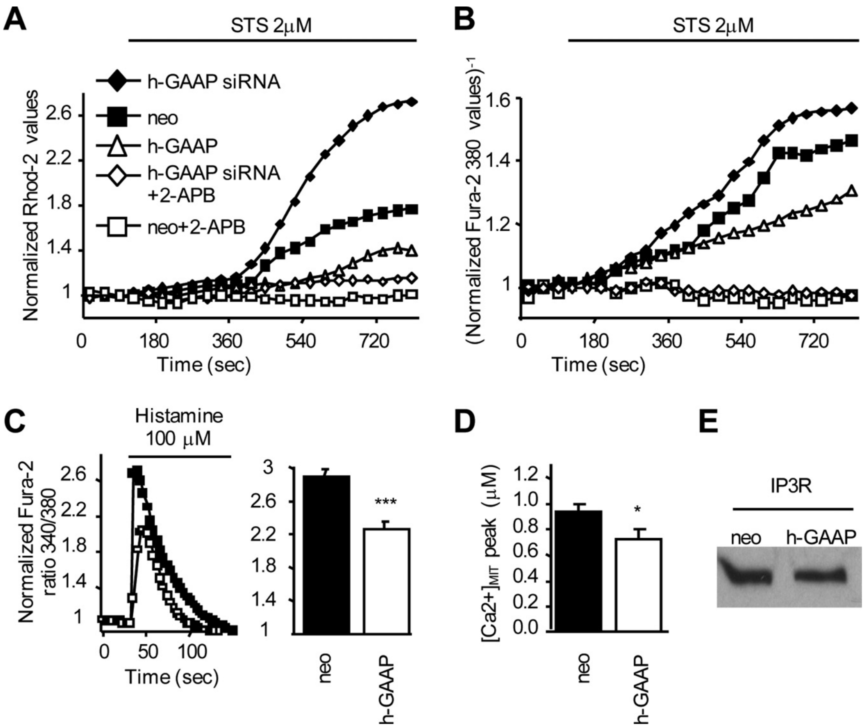

Figure 1. h-GAAP reduces staurosporine and histamine-induced Ca^{2+} rises in the cytosol and the mitochondria. (A and B) Fura-2 and rhod-2 coloaded cells were excited alternately at 380 and 540 nm for digital imaging microscopy of the STS (2 μM)-induced changes in rhod-2 (A) and fura-2 (B) fluorescence, respectively, in the absence or presence of 2-APB. STS was added at 120 s after the onset of monitoring. The fluorescence emission signal was normalized to its prestimulatory value. Typical records depicting changes in mitochondrial and cytosolic Ca^{2+} are shown (average of 13 cells is shown). In B, the scale representing the fura-2 380-nm fluorescence is inverted to give a more intuitive representation of the rise in cytosolic Ca^{2+}. (C) Fura-2–loaded cells seeded on glass coverslips were treated with 100 μM histamine and changes in cytosolic Ca^{2+} were monitored by digital imaging microscopy. In each experiment, the fluorescence emission ratio was normalized to its prestimulatory value, which was set at 1. Left, typical records depicting changes in cytosolic Ca^{2+} (average of 30–40 cells is shown); right, the average ± SEM of the peak amplitude from three independent experiments performed in duplicate. * $p < 0.05$, *** $p < 0.001$. (D) Luminescence analysis of histamine-induced changes in Ca^{2+} concentration in the mitochondrial matrix of neo and GAAP cells transfected with mitochondrion-targeted aequorin. * $p < 0.05$. (E) IP_3R expression levels in neo and h-GAAP cells lysates were determined by immunoblotting using an antibody recognizing all three IP_3R isoforms. Proteins were resolved by using 4–15% linear gradient SDS-PAGE.

itative entry of extracellular Ca^{2+} (Peppiatt *et al.,* 2003). Similarly, no increases were observed when using Ca^{2+}-free medium (data not shown). Importantly, expression of h-GAAP strongly reduced the STS-induced increase in both mitochondrial and cytosolic Ca^{2+} concentration.

To gain further support for a role of h-GAAP in regulating STS-induced Ca^{2+} fluxes, we next assessed the effect of h-GAAP down-regulation. Parental U2OS cells were transfected with siRNA1 (hereafter referred to as h-GAAP siRNA) or siRNA2 (hereafter referred to as control siRNA), shown before to decrease h-GAAP or be without effect on h-GAAP expression, respectively (Gubser *et al.,* 2007). Cells 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 Ca^{2+} 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 Ca^{2+} concentration was virtually completely inhibited by 2-APB. These results show that h-GAAP reduces the STS-induced increase in mitochondrial and cytosolic Ca^{2+} concentration, which depends on the influx of Ca^{2+} across the plasma membrane.

h-GAAP Decreases Histamine-induced Rises in Cytosolic and Mitochondrial Ca^{2+}

To gain more insight into a possible role of h-GAAP in the regulation of intracellular Ca^{2+} signaling, we investigated its effects on the increase in cytosolic and mitochondrial Ca^{2+} concentration evoked by the IP_3-generating hormone histamine. To prevent capacitative Ca^{2+} entry, stimulation with histamine was performed in the absence of extracellular Ca^{2+}, i.e., after dye-loading, cells were washed in Ca^{2+} free medium, transferred to Ca^{2+} 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 Ca^{2+} concentration, the amplitude of which was significantly reduced in h-GAAP cells (Figure 1C). Moreover, the upstroke of the Ca^{2+} 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 Ca^{2+} uptake, cells were transfected with a mitochondrial-targeted variant of the Ca^{2+}-sensitive photoprotein aequorin. Resting Ca^{2+} 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 Ca^{2+} concentration, the amplitude of which was significantly decreased in h-GAAP cells ($p < 0.05$; Figure 1D). Western blotting using an IP_3R antibody that recognizes all three subtypes revealed no detectable differences in the amount of IP_3R 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 Ca^{2+} concentration, mediated by IP_3Rs and, in this case, depending solely on the release of Ca^{2+} from intracellular stores.

h-GAAP Coimmunoprecipitates with the IP_3R

The above results suggested that h-GAAP might interact with IP_3Rs thus providing a potential explanation for its inhibitory effect on the flux of Ca^{2+} through these receptors. Because currently there exists no good antibody against h-GAAP for immunoprecipitation purposes, we made use of

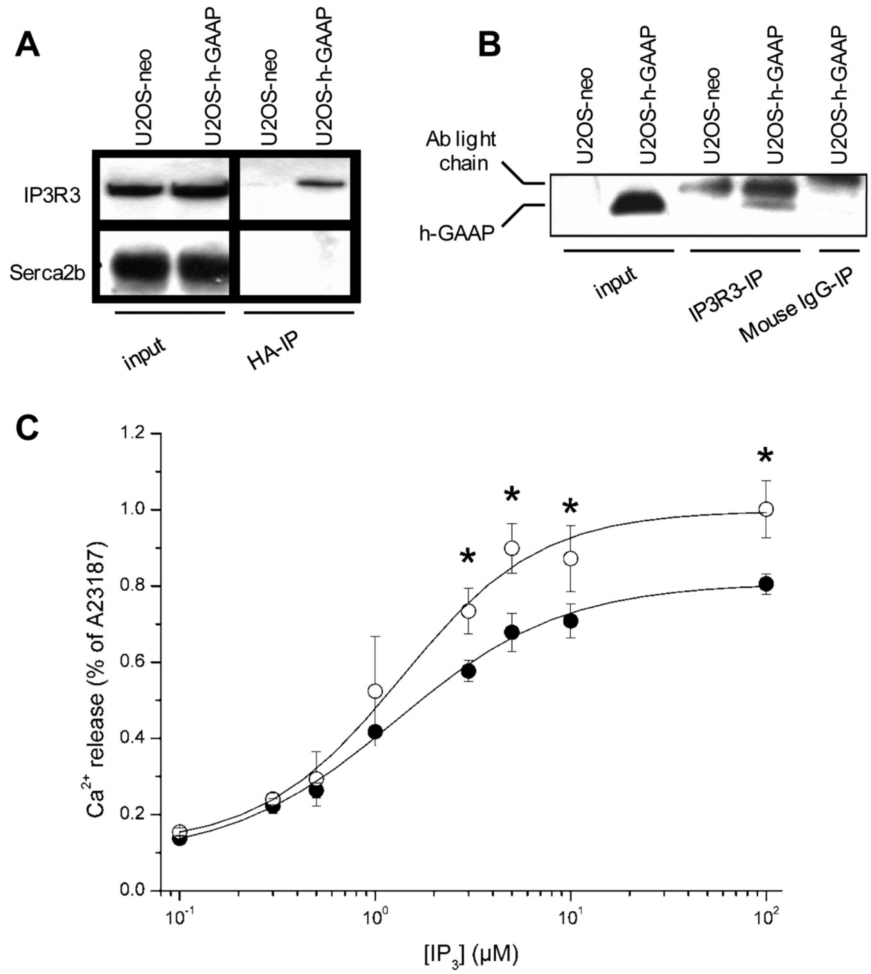

Figure 2. h-GAAP coprecipitates with IP_3R3, alters the sensitivity of the IP_3-induced Ca^{2+} release response and increases the passive Ca^{2+} leakage from the stores. (A) h-GAAP (HA-tagged) was immunoprecipitated from h-GAAP cells using an anti-HA mAb. Immunoprecipitates were analyzed by immunoblotting using anti-IP_3R subtype III (IP_3R3) or anti-SERCA 2B mAbs. (B) IP_3R3 was immunoprecipitated from neo and h-GAAP cells using an IP_3R3 mAb or a IgG control Ab (IgG-IP). Immunoprecipitates were analyzed by immunoblotting using an anti-HA mAb. (C) Permeabilized neo or h-GAAP cells were loaded with $^{45}Ca^{2+}$ for 45 min and then challenged with increasing concentrations of IP_3. The IP_3-induced Ca^{2+} release was counted, calculated relatively to maximal ionophore (A23187)-induced Ca^{2+} release, and plotted as a function of IP_3 concentration. The IP_3-insensitive Ca^{2+} passive leakage was subtracted from these values. Average values ± SD of three independent experiments performed in duplicate are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

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+}-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_3R, 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-

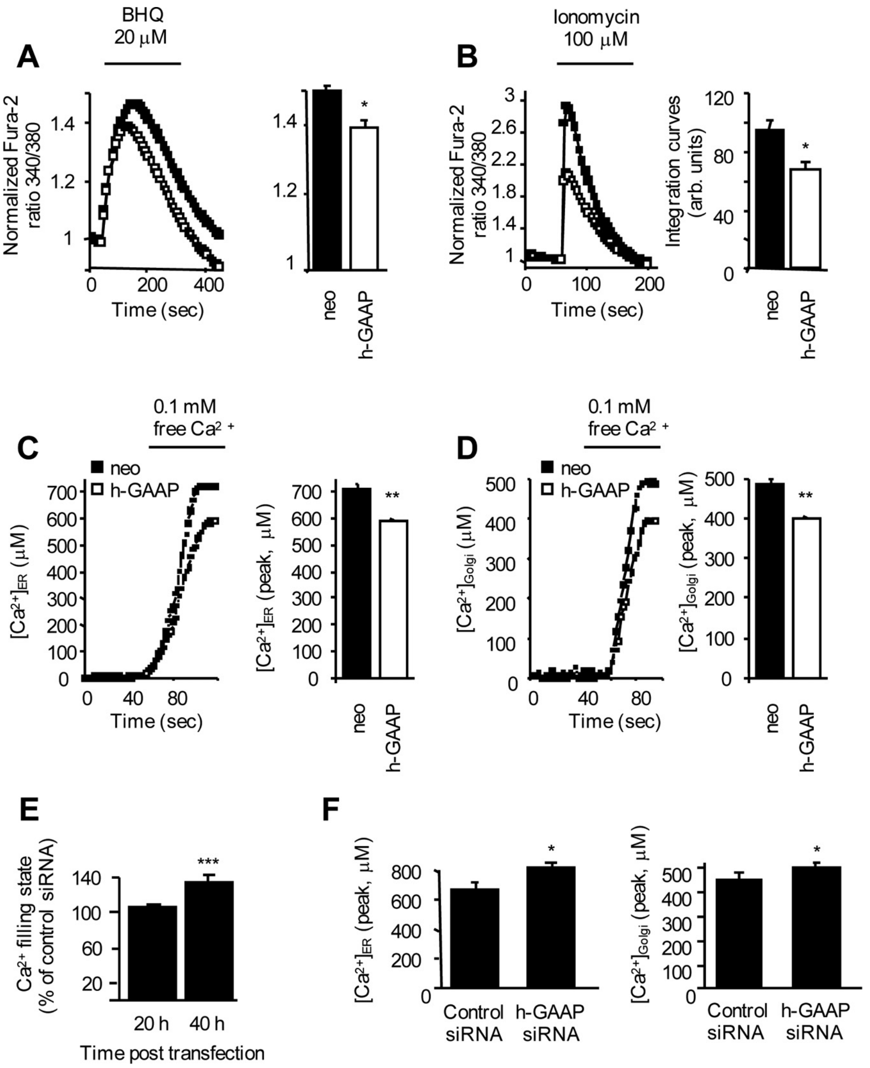

Figure 3. h-GAAP reduces the Ca^{2+} filling state of the intracellular stores. (A and B) Fura-2–loaded cells were treated either with 20 μM BHQ (A) or 1 μM ionomycin (B) and changes in cytosolic Ca^{2+} were monitored as described in Figure 2C. Right panels, the average ± SEM of the peak amplitude (A) 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. * $p < 0.05$.

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 μM. 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 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-

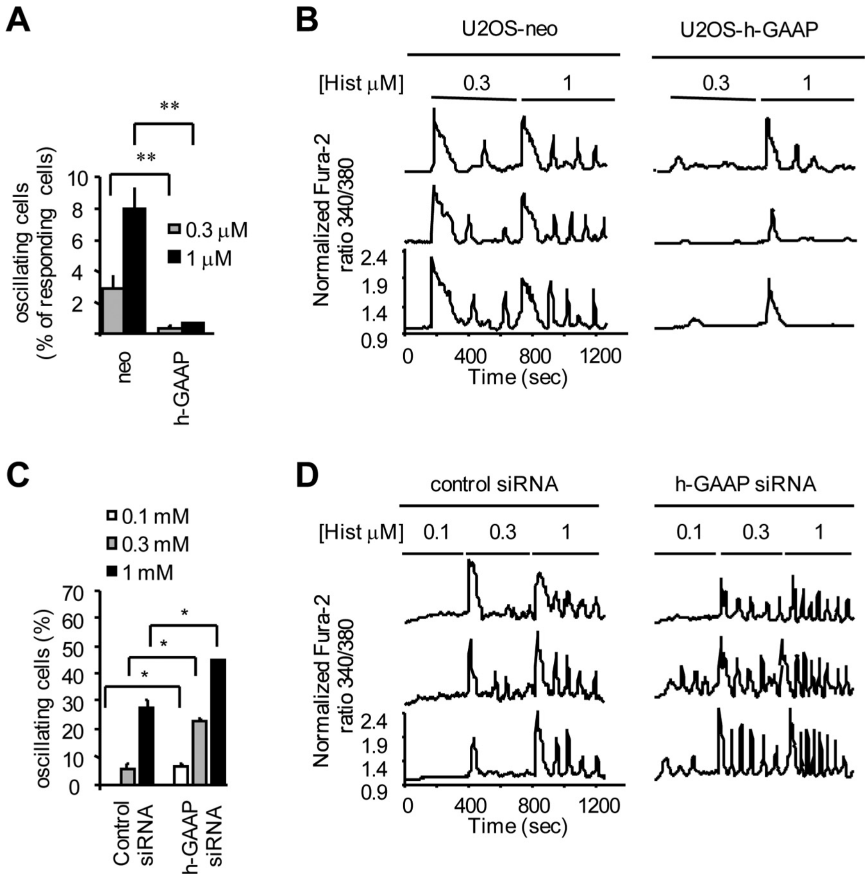

Figure 4. h-GAAP reduces the sensitivity to hormonal induction of cytosolic Ca^{2+} oscillations and reduces their frequency. (A and B) Fura-2–loaded cells were stimulated with different concentrations of histamine at the indicated time and monitored for their Ca^{2+} response. (A) Average percentage 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. * $p < 0.05$.

lations were observed only rarely in the h-GAAP cells, indicating that the occurrence of these oscillations is prevented in these cells. Taken together, these data are in agreement with the idea that h-GAAP reduces IP_3R activity in intact cells.

We also tested the effect of h-GAAP down-regulation on cytosolic Ca^{2+} signaling. Parental U2OS cells transfected with either h-GAAP siRNA or control siRNA were monitored for histamine-induced oscillatory Ca^{2+} changes at 2 d after transfection (Figures 4, C and D). About ~5 and ~30% of control siRNA-transfected cells, displayed oscillatory Ca^{2+} changes in response to 0.3 and 1.0 μM histamine, respectively. Similar percentages were observed in non-transfected parental U2OS cells (data not shown). Treatment with h-GAAP siRNA significantly increased the sensitivity of parental U2OS cells to histamine induction of oscillatory changes in cytosolic Ca^{2+} concentration (~25 and ~45% responding cells at 0.3 and 1.0 μM histamine, respectively; $p < 0.05$). Noticeably, a small number of cells responded already to a relatively low concentration of 0.1 μM histamine and, occasionally, even spontaneous Ca^{2+} oscillations were observed (data not shown).

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 overexpression 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_3Rs 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 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 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_3Rs, 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 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_3R-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_3R activity (by antisense knockdown, genetic deletion, or using a cell-permeable inhibitory peptide that interferes with the IP_3R-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_3R and alter its activity, though in opposite ways: Bcl-2 decreases the IP_3R 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 cytotoxic 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_3R 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_3R and modulates IP_3-induced Ca^{2+} signaling does not necessarily imply that it acts directly on the IP_3R. h-GAAP may be part of a larger IP_3R 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 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.

ACKNOWLEDGMENTS

F.J.M.v.K. is supported by grants from the Netherlands Organization for Scientific Research (NWO-VIDI-917.46.305) and the M.W. Beijerink Virology Fund from the Royal Netherlands Academy of Sciences. G.L.S. is a Wellcome Trust Principal Research Fellow. J.B.P. and H.de.S. were supported by research program G.0210.03 of the Fund for Scientific Research-Flanders and by Concerted Actions 04/07 of the Catholic University Leuven.

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