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Gas1 Interferes with A β PP Trafficking by Facilitating the Accumulation of Immature A β PP in Endoplasmic Reticulum-Associated Raft Subdomains

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Abstract

The amyloid- β protein precursor (A β PP) is a type I transmembrane protein that undergoes maturation during trafficking in the secretory pathway. Proper maturation and trafficking of A β PP are necessary prerequisites for A β PP processing to generate amyloid- β (A β), the core component of Alzheimer's disease senile plaques. Recently, we reported that the glycosylphosphatidylinositol (GPI)-anchored protein growth arrest-specific 1 (Gas1) binds to and interferes with the maturation and processing of A β PP. Gas1 expression led to a trafficking blockade of A β PP between the endoplasmic reticulum (ER) and the Golgi. GPI-anchored proteins can exit the ER by transiting through raft subdomains acting as specialized sorting platforms. Here, we show that Gas1 co-partitioned and formed a complex with A β PP in raft fractions, wherein Gas1 overexpression triggered immature A β PP accumulation. Pharmacological interference of ER to Golgi transport increased immature A β PP accumulation upon Gas1 expression in these raft fractions, which were found to be positive for the COPII protein complex component Sec31A, a specific marker for ER exit sites. Furthermore, a Gas1 mutant lacking the GPI anchor that could not transit through rafts was still able to form a complex with A β PP but did not lead to immature A β PP accumulation in rafts. Together these data show that Gas1 interfered with A β PP trafficking by interacting with A β PP to facilitate its translocation into specialized ER-associated rafts where immature A β PP accumulated.

Keywords

A β PP; Alzheimer's disease; (Gas1); lipid rafts; protein trafficking

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia and is due to progressive neurodegeneration in specific regions of the neocortex and hippocampus [1–3]. Two lesions are invariably found in the AD brain: the neurofibrillary tangles formed by tau deposition and senile plaques comprised of aggregated amyloid- β (A β) peptides [4, 5]. A β is produced by cleavage of the amyloid- β protein precursor (A β PP) via the sequential action of two

enzymes, β - and γ -secretases [6–9]. The identification of mutations in the A β PP gene causing familial AD has underscored the central role played by A β PP in AD pathogenesis [10, 11]. The trafficking of A β PP in the secretory pathway controls A β PP maturation in the endoplasmic reticulum (ER) and the Golgi before A β PP transport to the cell surface [12, 13]. Proper A β PP trafficking and maturation, via *O*- and *N*-glycosylation for instance, are necessary prerequisites for A β PP accessibility to the secretases at the cell surface or in the endosomal system and thus for the production of A β [14]. In this context, the different mechanisms controlling A β PP trafficking are under intense investigation.

To identify new genes potentially involved in AD pathogenesis, we previously used a tissue expression profiling strategy [15, 16] to screen for genes preferentially expressed in the hippocampus and located in AD chromosomal linkage regions. This approach identified the *CALHM1* (*calcium homeostasis modulator 1*) and *Gas1* (*growth arrest-specific 1*) genes [17]. We reported that CALHM1 codes for a novel calcium channel controlling cytosolic calcium homeostasis and A β metabolism [17]. Some studies indicated that CALHM1 may also control A β levels in human cerebrospinal fluid [18, 19] and influence AD age at onset [17, 20–22]. These results indicated that tissue expression profiling is a valuable approach for the identification of novel AD candidate genes.

Gas1 is a gene involved in the central nervous system development and in cell cycle control [23]. Gas1 codes for a glycosylphosphatidylinositol (GPI)-anchored protein required for normal postnatal proliferation in regions of the brain, such as the cerebellum [24–27]. Gas1 has also been associated with neuronal death in different models of excitotoxicity [28]. GPI-anchored proteins can exit the ER by transiting through specific coat protein complex II (COPII)-dependent raft subdomains acting as specialized sorting platforms at ER exit sites [29, 30]. These ER exit sites express specific protein markers, such as the COPII component Sec31A, and like plasma membrane lipid rafts, they are characterized by their resistance to non-ionic detergents. The detergent-resistant property of these subdomains allows their biochemical isolation in buffers containing Triton X-100 [29, 31].

We reported recently that, although Gas1 did not appear to represent an independent genetic determinant of AD risk from the available genome-wide association studies, Gas1 can form a complex with A β PP to control A β PP maturation and trafficking [32]. Gas1 expression was found to inhibit A β PP full glycosylation and to interfere with A β PP routing to the cell surface by leading to a trafficking blockade of A β PP between the ER and the Golgi. Consequently, Gas1 overexpression led to a reduction of A β production and conversely, Gas1 silencing in cells expressing endogenously Gas1 increased A β levels [32]. Here, we show that Gas1 interacted with A β PP in specific raft fractions, wherein Gas1 overexpression significantly facilitated the accumulation of immature A β PP. Using brefeldin A (BFA) treatments, we found that interference of ER to Golgi transport increased immature A β PP accumulation upon Gas1 expression in these raft fractions, which were found to be positive for the specific ER exit site marker, Sec31A. Furthermore, a GPI-free C-terminally truncated Gas1 mutant that could not transit through rafts was still able to form a complex with A β PP but did not lead to immature A β PP accumulation in rafts. Together these data confirm that Gas1 blocked A β PP trafficking between the ER and the Golgi and further show that Gas1 acted by interacting with A β PP to facilitate its translocation into specialized ER-associated raft subdomains where immature A β PP accumulated.

MATERIALS AND METHODS

Materials and antibodies

Methyl- β -cyclodextrin (MCD) was purchased from Sigma-Aldrich. BFA was from Epicentre Technologies. Anti-A β PP-(1–200) LN27 antibody was from Zymed Laboratories

and anti-A β -(17–24) 4G8 antibody was from Signet. The anti-A β PP C-terminal domain R1 antibody was provided by Dr. P.D. Mehta (Institute for Basic Research in Developmental Disabilities, Staten Island, NY). Antibodies directed against Gas1 were purchased from Santa Cruz Biotechnology (C-17, sc-9585) and Genetex Inc. (GTX101732). Anti-actin, anti-flotillin-1, and anti-Sec31A antibodies were from BD Transduction Laboratories. Anti-calreticulin (CRT) antibody was from ABR-Affinity Bio Reagents.

Cell culture, transfections, and western blot (WB) analyses

N2a cells stably transfected with human A β PP₆₉₅ harboring the Swedish double mutation (A β PP-N2a) and HEK293 cells stably transfected with human wild type A β PP₆₉₅ (A β PP-HEK293) were maintained in 1 : 1 Dulbecco's modified Eagle's medium (DMEM)/Opti-MEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were tested negative for mycoplasma contaminant [33]. Prior to transfection, cells were plated at a density of ~50%. Transient transfection (24 h) of Gas1 cDNA (cloned into pCMV6 expression vector, Origene) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. For WB, cells were washed with PBS and solubilized in ice-cold lysis buffer (25 mM HEPES, pH 7.4; 150 mM NaCl; 1% SDS; 1× Complete protease inhibitor mixture, Roche Applied Sciences). Cell extracts (5–20 μ g) were analyzed by SDS-PAGE using the antibodies listed above. A standard ECL detection procedure was then used.

Sucrose gradient centrifugation

A β PP-HEK293 cells in a 100-mm dish were washed and harvested in ice-cold PBS, and pelleted by centrifugation for 5 min at 1000 $\times$ g at 4°C. Cell pellets were lysed in 1.7 ml of MBS buffer (25 mM MES, pH 6.5; 150 mM NaCl; 1% CHAPS; 1× Complete protease inhibitor mixture), disrupted with 10 strokes in a glass Dounce homogenizer with a Teflon pestle, and sonicated 3 times for 5 s. The resulting cell lysates were mixed with 2.3 ml of MBS buffer containing 72% sucrose and added to the bottom of ultracentrifuge tubes. MBS buffer containing 35% sucrose (4 ml) was laid over the cell lysates, followed by 4 ml of MBS buffer containing 5% sucrose. Samples were centrifuged at 4°C for 16 h at 190,000 $\times$ g. Twelve fractions of 1 ml each were collected from the top to bottom of the ultracentrifuge tubes. An aliquot of each fraction was subjected to SDS-PAGE and WB analyses, as described above.

Preparation of Triton X-100-resistant membranes

Triton X-100-resistant membrane preparations were performed as described before [32]. Briefly, cells were lysed with Triton X-100 lysis buffer (TBS, 1% Triton X-100, 500 μ M sodium orthovanadate, 1× Complete protease inhibitor mixture) and under gentle shaking for 20 min at 4°C. The crude lysates were then centrifuged at 16,000 $\times$ g at 4°C for 10 min. The supernatants (Triton X-100-soluble fractions) were separated from the pellets and placed into separate tubes. The pellets were washed with ice-cold Triton X-100 lysis buffer and centrifuged again. Pellets (Triton X-100-resistant fractions) and Triton X-100-soluble fractions were then analyzed by WB.

Co-immunoprecipitation assays

Twenty-four hours post-transfection with pCMV6-Gas1 or empty vector, cells were washed with PBS and Triton X-100-resistant membranes were isolated as described above. Triton X-100-resistant membranes were resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.25% deoxycholic acid; 1% NP-40; 1% Triton X-100; 1 $\times$ Complete protease inhibitor mixture) and disrupted with 10 strokes in a glass Dounce homogenizer with a Teflon pestle and centrifuged at 16,000 $\times$ g for 5 min at 4°C. The

supernatants were pre-cleared with 50 μ l protein-G for 2 h at 4°C. After centrifugation at $1,500 \times g$ for 30 s, supernatants were incubated overnight at 4°C with the indicated antibodies. The following day, antibodies were precipitated by incubation with protein-G for 2 h at 4°C. Precipitates were washed 3 times with lysis buffer and then were resuspended in loading buffer (50 mM Tris, pH 6.8; 5% β -mercaptoethanol; 2% SDS; 0.1% bromophenol blue; 10% glycerol) and resolved by SDS-PAGE.

Statistical analysis

The results are presented as the mean $\pm$ standard deviation (S.D.). Significant differences between groups were determined using the unpaired Student's *t*-test. For all analyses, $p < 0.05$ was considered statistically significant.

RESULTS

Gas1 induces the accumulation of immature A β PP in rafts

Recently, we reported that Gas1 inhibited A β PP maturation and processing by leading to a trafficking blockade of A β PP between the ER and the Golgi [32]. Because Gas1, like many other GPI-anchored proteins, is known to localize in rafts, we asked whether Gas1 expression controls A β PP levels in rafts. Using sucrose gradient centrifugation, we observed a significant shift of A β PP into the flotillin-positive raft fraction in Gas1-transfected cells, as compared to control cells (Fig. 1A, fraction 5, and Fig. 1B). The effect of Gas1 expression on A β PP translocation into rafts was confirmed by another method using Triton X-100-resistant membrane preparations [34]. Similar to the observation using sucrose gradient centrifugation, Gas1 expression resulted in a robust accumulation of A β PP in flotillin-positive detergent-resistant raft fractions (Fig. 1C). Protein analysis using higher resolution WB showed that low-molecular-weight immature A β PP specifically accumulated in raft fractions upon Gas1 expression, whereas mature A β PP and A β PP C-terminal fragments, CTF α and CTF β , were detected at low levels in these fractions and were not significantly affected by Gas1 expression (Fig. 1D and E). Of note, Gas1 interfered with A β PP trafficking both in cells expressing wild type (A β PP-HEK293, Fig. 1A) and Swedish (A β PP-N2a, Fig. 1C) A β PP, suggesting that the Swedish mutation on A β PP did not impact Gas1 control of A β PP trafficking. Rafts and detergent-resistant membrane fractions are enriched in cholesterol and sphingolipids, two critical structural components for raft formation. Cholesterol depletion using methyl- β -cyclodextrin was found to lower the accumulation of both Gas1 and immature A β PP in detergent-resistant fractions (Fig. 1F and G), confirming that immature A β PP accumulated and co-partitioned with Gas1 in rafts in Gas1-transfected cells.

Gas1 and A β PP form a complex in rafts

Co-immunoprecipitation experiments previously revealed that Gas1 and A β PP can form a complex in cells [32]. Here, we asked whether this interaction takes place in rafts by performing reciprocal immunoprecipitations with antibodies directed against Gas1 or A β PP in detergent-resistant raft fractions resuspended in lysis buffer containing raft disrupting detergents. We found that Gas1 co-immunoprecipitated with A β PP in cells expressing the two proteins (Fig. 2A). These results indicated that Gas1/A β PP interaction can occur in rafts. We also observed that Gas1 preferentially co-precipitated with immature A β PP, suggesting that Gas1 interacted with A β PP early in the secretory pathway prior to A β PP maturation.

To confirm that Gas1 controls A β PP translocation into rafts, we asked whether inhibition of Gas1 trafficking in rafts affects A β PP accumulation in rafts upon Gas1 expression. Because incorporation of GPI-anchored proteins into rafts is selectively driven by their GPI anchor, a

Gas1 construct was generated in which the GPI consensus sequence was removed (Fig. 2B). We confirmed the inability of this GPI-free soluble Gas1 mutant (sGas1) to translocate into rafts (Fig. 2C). We found that GPI-free sGas1 was still able to form a complex with A β PP (Fig. 2D) but failed to trigger the accumulation of immature A β PP in rafts (Fig. 2E), showing that Gas1 translocation into rafts is required for its effect on A β PP accumulation in rafts. These results further demonstrate that the GPI anchor is dispensable for the Gas1/A β PP interaction.

Gas1 controls immature A β PP translocation into ER-associated rafts

Gas1 expression led to the accumulation of immature A β PP in rafts (Figs. 1 and 2), suggesting that the two proteins interacted early in the secretory pathway in a compartment close to the ER. Because GPI-anchored proteins can exit the ER by transiting through specific ER sorting platforms [29, 30], we next asked whether immature A β PP accumulates and co-partitions with Gas1 in ER-associated rafts. We previously reported that while the Triton X-100-soluble fractions are immunoreactive for calreticulin and therefore contained most of the ER, the Triton X-100-resistant fractions were found to be strongly immunoreactive for Sec31A and thus are enriched in ER exit sites, indicating that Gas1 may facilitate A β PP accumulation at these specific ER-associated subdomains [32]. To go further, we investigated whether interference of ER to Golgi transport prevents the effect of Gas1 expression on A β PP translocation into rafts. Treatments with BFA, an agent that blocks protein transport from the ER to the Golgi by disassembling the Golgi complex [35], failed to inhibit immature A β PP accumulation in raft fractions, which were found to be still positive for the ER exit site marker Sec31A and negative for the ER marker calreticulin (Fig. 3A and B). Instead, we found that BFA treatments slightly but significantly strengthened the effect of Gas1 expression on immature A β PP accumulation in rafts (Fig. 3A and B), confirming that A β PP accumulated in a compartment localized between the ER and the Golgi. Altogether these data show that Gas1 expression led to immature A β PP accumulation in ER-associated rafts.

DISCUSSION

Strong evidence indicates that lipid rafts are involved in A β PP amyloidogenic processing [36, 37]. Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains and studies in cell cultures and animal models have shown that alteration in cholesterol and sphingolipid distribution or metabolism can significantly impact A β PP processing and A β production [37, 38]. Both BACE1/ β -secretase and γ -secretase are enriched in lipid rafts and can target A β PP for A β production in these microdomains. γ -Secretase is involved in regulated intramembranous proteolysis of multiple type I transmembrane proteins beyond A β PP, such as Notch or N- and E-cadherins [8, 39]. γ -Secretase in the adult brain, however, appears to preferentially target A β PP in lipid rafts [40]. Together, these results motivate further studies aimed at determining whether targeting raft-dependent amyloidogenic processing of A β PP is therapeutically relevant.

Lipid raft biogenesis and cellular trafficking is complex. Most rafts are found at the plasma membrane where they follow the endocytotic pathway to recycle back to the plasma membrane or to return to the Golgi. Rafts found at the plasma membrane are mostly generated at the Golgi and use the anterograde pathway to traffic to the plasma membrane [41–43]. Some rafts, however, can be generated from the ER membrane and GPI-anchored proteins can use these rafts to exit the ER. These specific ER rafts are COPII-dependent subdomains acting as specialized sorting platforms [29, 30, 44]. Like plasma membrane lipid rafts, rafts at ER exit sites are characterized by their resistance to Triton X-100 at 4°C [29, 31]. At steady state, it is estimated that about 10% of A β PP is targeted to lipid rafts and that raft-localized A β PP is mostly regulated by endocytosis [45]. Here, we reveal another

level of complexity in A β PP trafficking in rafts. Using Triton X-100-resistant membrane preparations, we observed that Gas1 physically interacted with A β PP to co-partition in rafts at ER exit sites. It is important to note that the use of non-ionic detergents to isolate rafts and study protein trafficking in these microdomains has raised some concerns [46]. In this context, we also used a GPI-free Gas1 mutant that lost its properties to traffic through rafts to show that Gas1 translocation into rafts was required for its effect on A β PP accumulation in rafts. Thus, the current work shows that A β PP can also accumulate in specialized ER-associated rafts. Although further studies will be required to determine the relative amounts at steady state of A β PP transiting through these rafts and what might be the contribution of this pathway to constitutive A β PP processing, this work suggests that approaches aimed at increasing A β PP translocation in these ER-associated subdomains (e.g., by increasing Gas1 expression) can significantly influence A β PP maturation and processing, and thus A β production [32].

In summary, we provide strong evidence that Gas1 negatively controlled A β PP maturation by inhibiting A β PP trafficking between the ER and the Golgi and this by physically interacting with A β PP to trigger its translocation into specialized ER-associated raft subdomains where immature A β PP accumulated. This study reveals a new facet of A β PP trafficking and further increases our understanding of Gas1 function.

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Fig. 1.

Gas1 induces the accumulation of immature AβPP in rafts. A) AβPP-HEK293 cells transiently transfected with Gas1 cDNA or empty vector were lysed in 1% CHAPS buffer and subjected to discontinuous sucrose density gradient ultracentrifugation fractionation. All fractions (1–12) were analyzed by WB using antibodies directed against AβPP, Gas1, calreticulin (CRT, ER resident protein), and flotillin-1 (lipid raft resident protein). B) Shown are densitometric analysis and quantification of AβPP immunoreactivity in fraction 5 (raft AβPP) in three independent experiments performed as in A. C) AβPP-N2a transiently transfected with Gas1 cDNA (+) or empty vector (–) were lysed in 1% Triton X-100 buffer at 4°C and Triton X-100-soluble and Triton X-100-resistant fractions (see Materials and Methods) were analyzed by WB for the indicated proteins. D) WB analysis of protein extracts from cells treated as in C showing the accumulation of immature AβPP in Triton X-100-resistant fractions upon Gas1 transfection. *ma. AβPP*, mature AβPP; *im. AβPP*, immature AβPP. E) Shown are densitometric analysis and quantification of immature and mature AβPP levels in three independent experiments performed as in D. F) AβPP-HEK293 cells transiently transfected with Gas1 cDNA (+) or empty vector (–) were treated (+) or not (–) with methyl-β-cyclodextrin (MCD, 10 mM, 1 h). Triton X-100-soluble and Triton

X-100-resistant fractions were then analyzed by WB using antibodies directed against the indicated proteins. Note that the effect of MCD on raft integrity was confirmed by the partial translocation of flotillin in the Triton X-100-soluble fractions (3rd and 4th lanes). G) Shown are densitometric analysis and quantification of immature A β PP levels in Triton X-100-resistant fractions in three independent experiments performed as in F. Histograms in B, E, and G indicate the mean $\pm$ S.D. *a.u.*, arbitrary units. * p < 0.05, ** p < 0.01 (Student's *t*-test).

Fig. 2.

Gas1 and AβPP form a complex in rafts. A) Triton X-100 resistant fraction homogenates from AβPP-HEK293 cells transiently transfected with Gas1 cDNA (+) or empty vector (-) were incubated in the absence (CTRL) or presence of anti-AβPP (4G8) or anti-Gas1 (C-17) antibodies. Total extracts from Triton X-100 resistant fractions (Input) and immunoprecipitated (IP) proteins were analyzed by WB using antibodies directed against AβPP (LN27) and Gas1 (GTX101732). B) Map of the constructs generated to express Gas1 full-length and soluble Gas1 (sGas1) lacking the GPI consensus sequence. C) AβPP-N2a cells transiently transfected (+) or not (-) with Gas1 or sGas1 cDNAs were lysed in 1% Triton X-100 buffer at 4°C and Triton X-100-soluble and Triton X-100-resistant fractions were analyzed by WB for the indicated proteins. D) AβPP-HEK293 cells transiently transfected with sGas1 cDNA (+) or empty vector (-) were IP with anti-AβPP (4G8) antibody. Total extract (Input) and precipitated proteins were analyzed by WB using antibodies directed against AβPP (LN27), Gas1 (GTX101732), and actin. E) AβPP-HEK293 cells transiently transfected with empty vector, Gas1 cDNA, or sGas1 cDNA were lysed in 1% Triton X-100 buffer at 4°C. Triton X-100-soluble and Triton X-100-resistant fractions were then analyzed by WB for the indicated proteins.

Fig. 3.

Gas1 controls immature AβPP translocation into ER-associated rafts. A) AβPP-N2a cells transiently transfected with Gas1 (+) or empty vector (−) were treated with brefeldin A (BFA, 5 μg/ml) for the indicated times. Cells were then lysed in 1% Triton X-100 buffer at 4°C and Triton X-100-soluble and Triton X-100-resistant fractions were analyzed by WB for the indicated proteins. B) Shown are densitometric analysis and quantification of immature and mature AβPP levels in three independent experiments performed as in A. Histogram indicates the mean ± S.D. *a.u.*, arbitrary units. **p* < 0.05, ***p* < 0.01 (Student's *t*-test).