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γ-secretase activating protein (GSAP) does not specifically affect the γ-secretase processing of APP

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Abstract
Alzheimer's disease (AD) is characterized by the cerebral accumulation of β-amyloid (Aβ) peptide, which is generated by proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases (Vassar J Mol Neurosci, 2004). γ-Secretase is an attractive therapeutic target for the treatment of AD; however, it also cleaves several other protein substrates including Notch (Sisodia et al., Nature Rev Neuro, 2002). A recent study reported a novel γ-secretase activating protein (GSAP) that is processed from a holoprotein to a 16 kDa active form, which interacts with the γ-secretase complex to selectively regulate β-amyloid peptide generation from APP, without affecting Notch [1]. Since this finding implies that the GSAP processing might be an important target for AD, we tested if this finding could be reproducible. Here we show that processing of GSAP is not required for Aβ production, and rather than specifically regulating γ-secretase cleavage of APP, GSAP also regulated the levels of sAPPβ, the ectodomain shedded by the β-secretase processing of APP. Thus, our results caution the validity of GSAP as the γ-secretase-specific therapeutic target for Aβ production in AD.

Introduction
γ-Secretase is an attractive therapeutic target for the treatment of Alzheimer's disease (AD); however, it also cleaves several other protein substrates including Notch. γ-Secretase is a multimeric transmembrane protein complex composed of presenilin-1 (PS1)/presenilin-2 (PS2), Nicastrin, Aph-1, and Pen2 [2]. Familial mutations in APP, PS1, or PS2 that increase the production of the amyloidogenic Aβ42 peptide have been associated with early-onset AD [3] [4]. Thus, γ-secretase is an attractive therapeutic target for AD. However, γ-secretase inhibition affects the cleavage of physiologically important substrates such as Notch, and also the ε-cleavage of APP that releases the APP intracellular domain (AICD). The failure of recent clinical trials with γ-secretase inhibitors highlights the need for Aβ-specific inhibitors that spare the cleavage of Notch and the release of AICD [5] [6] [7]. Recent studies discovered that an anti-cancer compound, imatinib (also known as Gleevec), inhibited Aβ peptides without affecting AICD cleavage. In a hunt for the mechanism through which imatinib produced this effect, He et al. identified a novel γ-secretase activating protein (GSAP) [7]. The authors demonstrated that GSAP is synthesized as a holoprotein called pigeon homologue protein (PION) that is readily processed to a 16 kDa fragment. The authors further showed that this 16 kDa GSAP is the predominant form under steady-state conditions, which interacted with γ-secretase to modulate Aβ production. They also showed that overexpression of GSAP increased, whereas its silencing decreased, Aβ levels. Since this finding implies that the GSAP processing might be an important target for AD, we tested if this finding could be reproducible so that GSAP can be established as a reliable target for the treatment of AD.

Objective
Here we wanted to study if processing of GSAP is required for Aβ production, and if GSAP specifically affects the γ-secretase processing of APP.

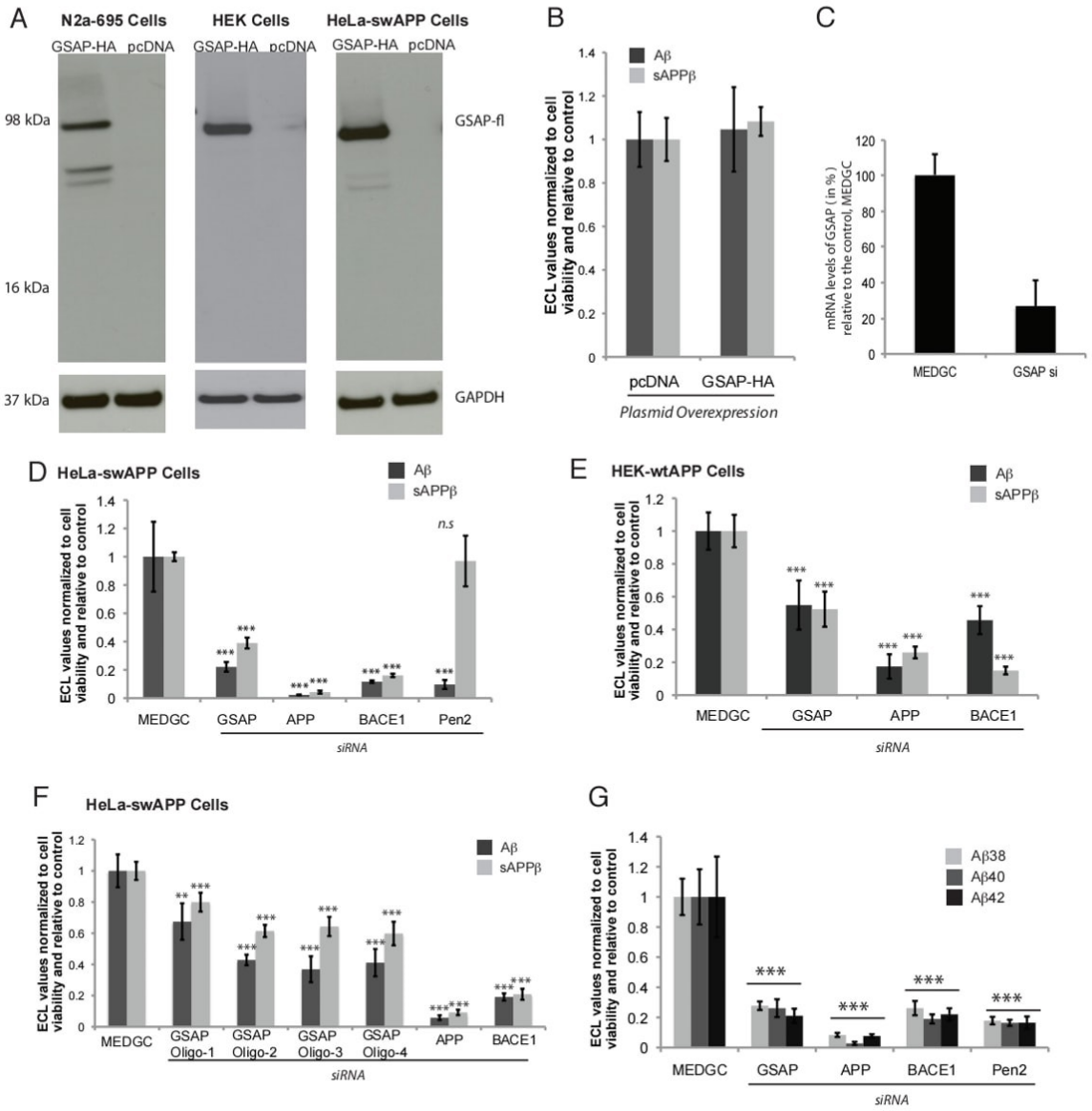

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Figure Legend

Figure 1. Full-length γ-secretase activating protein (GSAP) is not processed to a 16 kDa protein and its silencing leads to reduction in both β- and γ-secretase cleavage of APP.

(A) Western blot analysis showing absence of the processed 16 kDa C-terminal fragment of GSAP: N2a-695 cells, HEK cells and HeLa-swAPP cells were transfected with either the pReceiverM07 construct expressing the full-length GSAP with a C-terminal-HA tag or pcDNA as the control plasmid and immunoblotted with anti-HA and anti-GAPDH antibodies.

(B) Graph showing the levels of Aβ and sAPPβ after overexpression of GSAP-HA plasmids: HeLa-swAPP cells were transfected with pcDNA (negative control) or the plasmid expressing GSAP, and the supernatants were analyzed for sAPPβ and Aβ levels using an electrochemiluminescence (ECL)-based assay.

(C) Graph showing GSAP knockdown efficiency: HeLa-swAPP cells were transfected with either MEDGC oligos (negative control) or siRNAs against GSAP and the knock-

down efficiency was estimated by quantitative RT-PCR.
(D) Graph showing the reduced levels of Aβ and sAPPβ after siRNA-mediated silencing of GSAP in Hela-swAPP cells: HeLa-swAPP cells were transfected with MedGC oligos (negative control) and with siRNA pools for silencing the expression of APP, BACE1, PEN2 and GSAP, and assayed for Aβ and sAPPβ using ECL.
(E) Graph showing the reduced levels of Aβ and sAPPβ after siRNA-mediated silencing of GSAP in HEK-wtAPP expressing cells: HEK-wtAPP cells were transfected with MedGC oligos and siRNAs against APP, BACE1, and GSAP and assayed for Aβ and sAPPβ.
(F) Graph showing the levels of Aβ and sAPPβ after siRNA-mediated silencing of GSAP using four different GSAP-specific siRNAs: HeLa-swAPP cells were transfected with MedGC oligos and siRNAs against APP, BACE1, and four different siRNAs against GSAP and assayed for Aβ and sAPPβ.
(G) Graph showing the levels of Aβ38, Aβ40 and Aβ42 after siRNA-mediated silencing of GSAP: HeLa-swAPP cells were transfected with MedGC oligos and siRNAs against APP, BACE1, PEN2, and GSAP and assayed for Aβ38, Aβ40, and Aβ42.
For Fig. 1D–1F and Fig. 1I–1K, $^{**}p < 0.05$, $^{***}p < 0.005$, n.s., not significant. All statistics are performed using a two-tailed t-test. Error bars indicate SD.

cDNA constructs

For GSAP overexpression, mammalian expression vector pReceiverMo7 with the full-length GSAP and a C-terminal-HA tag was purchased from Genecopoeia (same source as He et al.).

Cells

Hela-sweAPP and HEK-wtAPP cells are cultured and used as described 20. N2a-695-APP cells are a kind gift of Prof. G. Thinakaran, University of Chicago.

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siRNA transfection of HEK-wtAPP cells

Lipofectamine RNAiMAX (Invitrogen) was used as a transfection reagent. 24 h prior to transfection, cells were seeded at an initial seeding density of 3500 cells per well in a 96 well plate pre-coated with poly D lysine (Sigma Aldrich). siRNAs were transfected at a final concentration of 5 nM using 0.3 µl Lipofectamine RNAiMAX in a total volume of 100 µl. Each siRNA transfection was performed in quadruplicate. After 48 h the transfection mix was replaced with fresh medium containing 10% Alamar Blue. Supernatants were collected and assayed for Aβ and sAPPβ as described below. The cells were lysed with 35 µl lysis buffer for 20 min on ice.

Plasmid transfection

The plasmid transfection for GSAP/PION-HA was performed in HeLa-swAPP cells. Lipofectamine 2,000 (Invitrogen) was used as a transfection reagent. Cells were seeded in a 96 well plate at an initial seeding density of 6,000 cells per well 24 h prior to the transfection. 0.3 µg of plasmid DNA was transfected using 0.3 µl of Lipofectamine 2,000 in a total volume of 100 µl. Transfection mix was replaced after 3 h by fresh culture medium. 21 h after transfection, medium was again replaced with fresh medium containing 10% Alamar Blue. 24 h after transfection, Alamar Blue measurements were taken. Supernatant was collected and assayed for Aβ and sAPPβ. The cells were lysed with 35 µl lysis buffer, incubated for 20 min on ice, and stored at -20°C.

Alamar Blue assay

For cell viability measurements using Alamar Blue, the medium of transfected cells (siRNA or plasmid) was replaced with normal medium containing 10% Alamar Blue. The final volume in each well was 100 µL. 3 h after medium change, cell viability was monitored using Fluoroscan Ascent Cf (Labsystems), with excitation wavelength 544 nm and emission at 590 nm. Cell viability was measured using the Alamar Blue assay (Serotec Ltd., Kidlington, Oxford, UK), where the absorbance was monitored at the end of the reaction (after 3 h) (Labsystems Multiscan MS UV visible spectrophotometer).

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Protease prediction tool

We used the Swiss Institute of Bioinformatics' Expassy Peptide cutter tool (http://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl). We pasted the human GSAP sequence (UniProtKB/Swiss-Prot: A4D1B5.2) into the field and chose both enzymes and chemicals that could cleave from 1 up to 20 cleavage sites. The following sequence was provided:

>gi|189041192|sp|A4D1B5.2|GSAP_HUMAN RecName: Full=Gamma-secretase-activating protein; Short=GSAP; AltName: Full=Protein pigeon homolog; Contains: RecName: Full=Gamma-secretase-activating protein 16 kDa C-terminal form; Short=GSAP-16K MALRLVADFDLGKDVLPWL-RAQRAVSEASGAGSGGADVLENDYESLHVLNVERNGNIIYTYKDDKGNVVFGLY-DCQTRQNELLYTFEKDLQVFSCSVNSERTLLAASLVQSTKEGKRNELQPGSKCLTL-LVEIHPVNNVKVLKAVDSYIWVQFLYPHIESHPLPENHLLLISEEKYIEQFRIH-VAQEDGNRVVIKNSGHLPRDRIAEDFVWAQWDMSEQRLYYIDLKKSRSILKCIQ-FYADESYNLMFEVPLDISLSNSGFKLVNFGCDYHQYRDKFSKHLTLCVFTNHT-GSLCVCYSPKCASWGQITYSVFYIHKGHSKTFTTSLENVGSHMTKGITFLNLDYY-VAVYLPGHFFHLLNVQHPDLICHNLFLTGNNEMIDMLPHCPLQSLSGSLVLDCCS-GKLYRALLSQSSLLQLLQNTCLDCEKMAALHCALYCGQGAQFLEAQIIQWISEN-VSACHSFDLIQEFIIASSYWSVYSETSNMDKLLPHSSVLTWNTEIPGITLVTEDI-ALPLMKVLSFKGYWEKLNSNLEYVKYAKPHFHYNNSVVRREWHNLISEEKT-GKRRSAAYVRNILDNAVKVISNLEARNLGPRLTPLLQEEDSHQRLLMGLMVSELKD-HFLRHLQGVEKKKIEQMVLDYISKLLDLICHIVETNWRKHNLHSWVLHFNSRGSAAE-FAVFHIMTRILEATNSLFLPLPPGFHTLHTILGVQCLPLHNLLHCIDSGVLLLTE-

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LLQNYKKQPRNSMINKSSFSVEFLPLNYFIEILTDIESSNQALYPFEGHDNVDAE-
FVEEAALKHTAMLLGL

Results & Discussion

Since GSAP has been shown to be processed to a 16 kDa fragment by an unknown protease and since this protease could be an ideal therapeutic target for AD [8], we intended to perform a protease RNA interference (RNAi) screen to identify the protease(s) responsible for the cleavage of GSAP. To establish conditions for detecting GSAP cleavage, we first expressed full-length GSAP (also called PION) in cells that produced robust amounts of Aβ [9]. Unexpectedly, we did not detect any 16 kDa cleavage product of GSAP/PION, and the full-length levels of GSAP/PION remained largely unaltered in all the cells that we tested, namely N2a cells expressing the Swedish mutant of APP (N2a-695), HEK cells and HeLa cells expressing the Swedish mutant of APP (HeLa-swAPP) cells (Fig. 1A) in contrast to the findings of He et al. We used the GSAP construct that He et al. used but failed to see any cleavage of GSAP. We also used bioinformatics analysis to predict protease cleavage sites in GSAP and used the sequence of human GSAP as entry in Expassy's Peptide Cutter tool, a standard protease prediction online tool. First, there were only two enzymes that were predicted to cut GSAP once, but neither of them would liberate a 16 kDa fragment (Caspase 1 at residue 8 and Thrombin at residue 591) (see Suppl. Fig. 1). Second, even when we relaxed the number of times that the enzymes (and also chemicals) could cleave GSAP to up to 20 sites, there were no cleavage sites at the residue 733 predicted by He et al., to release a 16 kDa fragment, suggesting that GSAP is most likely not processed to a 16 kDa fragment by a cellular protease, one of the two core findings of He et al., i.e., GSAP is processed to a 16 kDa fragment that binds to γ-secretase and it is a γ-secretase activating protein, which implied that this protease could be a therapeutic target. Importantly, even in the absence of proteolytic processing of GSAP, the cells still produced robust amounts of Aβ suggesting that cleavage of GSAP is not essential for its putative γ-secretase-promoting activity (Fig. 1B).

To study the role of GSAP in APP processing and Aβ production, we silenced GSAP using a pool of four siRNAs and assayed for secreted Aβ and sAPPβ levels, the soluble fragment of APP that is generated after β-secretase-(BACE1)-mediated cleavage. As controls, we used siRNAs against APP, BACE1, Pen2, and a scrambled medium GC oligo (MedGC). RT-PCR analyses confirm efficient silencing of GSAP upon siRNA transfection (Fig. 1C). The measurements to monitor APP processing were performed using a multiplexed system to quantitatively measure both Aβ and sAPPβ levels from the sample [10]. This system allows one to investigate if a particular perturbation (via siRNAs or plasmid overexpression) affects at the level of β-cleavage of APP or at the level of γ-secretase/Aβ . As reported by He et al., we found that GSAP silencing significantly inhibited Aβ levels (Fig. 1D). However, we also found a similar decrease in sAPPβ levels (Fig. 1D). Since both the measurements come from the same samples and even from the same wells of the assay plates, the decrease in both sAPPβ and Aβ were not due to inter-measurement variations or use of different assays [10]. This decrease in both sAPPβ and Aβ was unexpected as it was claimed that GSAP regulated only γ-secretase cleavage of APP (He et al.). To ensure that our assay can recapitulate specific γ-secretase-affecting events, as a control, we silenced the expression of Pen2, a bona fide γ-secretase associated/activating protein [11], and found that it affected specifically Aβ levels without affecting sAPPβ levels (Fig. 1D). As additional controls, we silenced the expression of APP and BACE1, which reduced both sAPPβ and Aβ levels as expected (Fig. 1D). siRNA-mediated silencing of genes works very efficiently in these cells ([12]Fig. 1C). Similar results were obtained when GSAP was silenced in HEK cells, which express wildtype APP (Fig. 1E). To rule out that the effect on sAPPβ was caused by off-target effects through siRNA silencing with pooled siRNAs, we silenced GSAP using four independent siRNA oligos individually and assayed for sAPPβ and Aβ levels. All individual siRNAs decreased both sAPPβ and Aβ, clearly demonstrating that GSAP regulates both sAPPβ and Aβ levels, and not just the γ-cleavage product, Aβ (Fig. 1F), as reported by He et al. The probability that all four oligos would work through an off-target is infinitesimally small and this clearly demonstrates that GSAP affects both Aβ and sAPPβ levels. Moreover, using the multiplexing system for measuring the peptides Aβ38, 40

and 42 [12] we found that all three Aβ species were significantly reduced upon GSAP knockdown (Fig. 1G).

Conclusions

We show that GSAP is not processed to a 16 kDa fragment and it does not specifically alter γ-secretase cleavage of APP as claimed by He et al.

Limitations

One main limitation that we can think of is that our study used only cultured cells, but we used three different systems and found exactly the same result: that we could not replicate the He et al. studies, in line with the findings from Hussain et al. [1].
Since GSAP silencing also reduced the levels of sAPPβ in addition to Aβ, it could affect either the levels of APP or β-secretase (BACE) (in the case of β-secretase it could also affect the activity). In the next studies, we will follow up by studying whether GSAP regulated the levels/activity of APP/BACE1. If other labs are also interested, we invite them so that they can also investigate to know how GSAP affected sAPPβ and Aβ levels. We will provide the reagents we have used here.

Additional Information

Methods

cDNA constructs

For GSAP overexpression, mammalian expression vector pReceiverMo7 with the full-length GSAP and a C-terminal-HA tag was purchased from Genecopoeia (same source as He et al.).

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FAVFHIMTRILEATNSLFLPLPPGFHTLHTILGVQCLPLHNLLHCIDSGVLLLTE-
TAVIRLMKDLDNTEKNEKLKFSIIVRLPPLIGQKICRLWDHPMSSNIISRNHVTR-
LLQNYKKQPRNSMINKSSFSVEFLPLNYFIEILTDIESSNQALYPFEGHDNVDAE-
FVEEAALKHTAMLLGL

Supplementary Material

Please see https://sciencematters.io/articles/201511000002.

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Ethics Statement

Not applicable.
All experiments were done in accordance with the standard guidelines.

Citations

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