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Purification and Characterization of the Human γ-Secretase Activating Protein

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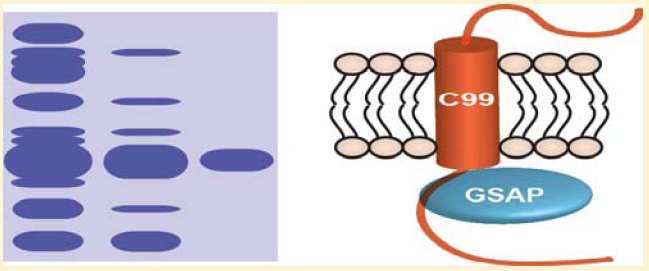
Supporting Information

ABSTRACT: Alzheimer's disease is a fatal neurological disorder that is a leading cause of death, with its prevalence increasing as the average life expectancy increases worldwide. There is an urgent need to develop new therapeutics for this disease. A newly described protein, the γ-secretase activating protein (GSAP), has been proposed to promote elevated levels of amyloid-β production, an activity that seems to be inhibited using the well-establish cancer drug, imatinib (Gleevec). Despite much interest in this protein, there has been little biochemical characterization of GSAP. Here we report protocols for the recombinant bacterial expression and purification of this potentially important protein. GSAP is expressed in inclusion bodies, which can be solubilized using harsh detergents or urea; however, traditional methods of refolding were not successful in generating soluble forms of the protein that contained well-ordered and homogeneous tertiary structure. However, GSAP could be solubilized in detergent micelle solutions, where it was seen to be largely α-helical but to adopt only heterogeneous tertiary structure. Under these same conditions, GSAP did not associate with either imatinib or the 99-residue transmembrane C-terminal domain of the amyloid precursor protein. These results highlight the challenges that will be faced in attempts to manipulate and characterize this protein.



Alzheimer's disease (AD) is a devastating neurodegenerative disease that impacts millions of people worldwide at enormous personal and economic cost.[1] Unfortunately, there is currently no cure or effective treatment, but researchers have made significant progress in characterizing the pathophysiology of AD.[2] The most widely accepted hypothesis for the etiology of the disease revolves around the amyloid precursor protein (APP).[3] APP is cleaved by β-secretase to generate its 99-residue transmembrane C-terminus (C99), which is then cleaved by γ-secretase to produce amyloid-β (Aβ) peptides of different lengths. These peptides form neurotoxic oligomers that are then deposited as neuritic plaques, the pathological markers of the disease.

Inhibition of the heterotetrameric γ-secretase to block cleavage of C99 would reduce the level of Aβ production.[4−6] Unfortunately, γ-secretase has numerous substrates and has not, so far, been an effective therapeutic target because of the important role that its cleavage of other substrates, particularly Notch, plays in cellular biology.[7] As such, there is great interest in exploring how to prevent or modulate C99 cleavage without inhibiting cleavage of other γ-secretase substrates. This imperative resulted in the discovery of the γ-secretase activating protein (GSAP).

GSAP was first described by He et al.[8] A previous study had shown that the Abl kinase inhibitor imatinib decreases the level of Aβ production, likely by inhibiting γ-secretase activity.[9] The search for the imatinib target led to photolabeling of the C-terminal domain of the uncharacterized pigeon homologue protein (PION). The domain is proteolytically released from PION under cellular conditions, the resulting protein being termed GSAP. GSAP appears to form a ternary complex with γ-secretase and C99, as determined through immunoprecipitation reactions and pull-down assays. Knockdown of GSAP through siRNA in N2a cells selectively lowered Aβ levels and did not reduce the level of cleavage of other γ-secretase substrates. GSAP knockdown also reduced the Aβ plaque burden in a mouse model of AD.[8] These data suggest that GSAP may selectively promote Aβ production by promoting γ-secretase cleavage of C99, making GSAP a potential AD drug target. Since the initial discovery of GSAP, one additional research paper has been published; it characterized the immunohistochemical distribution of GSAP in the brains of AD patients.[10] GSAP immunoreactivity was observed in four distinct morphological structures present in different regions of the brain in AD patients; one of these structures was largely unique to AD brains as compared to age-matched control brains. GSAP immunoreactivity was also detected in the proximity of presenilin (PS1, a component of γ-secretase) as well as in association with Aβ-containing senile plaques. While recombinant expression and purification of GSAP were briefly mentioned in these reports, methods were not provided. This

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paper details the expression, purification, and characterization of GSAP.

MATERIALS AND METHODS

Materials. BL21(DE3) and Rosetta(DE3) competent cell lines and the pET32a vector were purchased from EMD Millipore (Darmstadt, Germany). The restriction enzymes NdeI, XhoI, BamI, and NcoI were purchased from New England Biolabs (Ipswich, MA). The $^{15}NH_4Cl$ used to isotopically label GSAP was purchased from Cambridge Isotope Laboratories (Andover, MA). Ampicillin and the MEM vitamin solution were purchased from Cellgro (Manassas, VA). Imatinib mesylate was purchased from Selleck Chemical Co. (Houston, TX). Ni-NTA chromatography resin was purchased from Qiagen (Valencia, CA). The protease inhibitor P8849, Empigen BB detergent (*n*-dodecyl *N*,*N*-dimethylglycine), and imidazole (≥99% titration grade) were purchased from Sigma-Aldrich (St. Louis, MO). *n*-Dodecylphosphocholine (DPC), lyso-myristoylphosphatidylglycerol (LMPG), and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Affymetrix/Anatrace (Maumee, OH).

Cloning and Construction of the Vectors Encoding His-Tagged Forms of GSAP. GSAP corresponds to the 121-residue (amino acids 733−854) C-terminus of the human pigeon homologue protein (PION). The GSAP gene (NM_017439.3) was purchased from GeneCopoeia (Rockville, MD). Two constructs were prepared, with either N- or C-terminal His purification tags (His_6 or His_{10}, respectively). To construct the C-terminally His_{10}-tagged construct, the GSAP DNA was digested with NdeI and XhoI after PCR amplification and was then inserted into the pET-21b vector. The N-terminal His_6-tagged construct was similarly engineered using NcoI and XhoI restriction enzymes and a pET-16a vector. Constructs were confirmed by DNA sequencing.

A second set of constructs was prepared to replicate as closely as possible the constructs used by He et al.[8] The first was a pET-32a vector encoding a fusion protein in which thioredoxin is linked to the N-terminus of GSAP through an intervening His_6 tag. As with the His_6-tagged constructs expressing only GSAP, the construct encoding the thioredoxin fusion protein was constructed from the DNA digested with BamI and XhoI after PCR amplification of the GSAP gene and was then inserted into the pET-32a vector. A second construct, expressing only thioredoxin, was prepared as a control by insertion of a stop codon just before the start of the GSAP coding region in a pET-32a vector. This was accomplished using standard site-directed mutagenesis methods (QuickChange, Agilent Technologies, Santa Clara, CA).

Expression of GSAP in *Escherichia coli*. Vectors were transformed into *E. coli* BL21(DE3) cells, which were plated onto ampicillin LB-agar plates and then incubated overnight at 37 °C. A single colony was used to inoculate a 5 mL culture of LB medium containing 100 μg/mL ampicillin. The starter culture was grown for 8 h at 37 °C. A 1 L culture of M9 minimal medium was prepared using $^{15}NH_4Cl$ for isotopic labeling. The medium for large-scale growth also included ampicillin, glucose, MEM vitamins, 0.1 mM $CaCl_2$, and 1 mM $MgSO_4$. A starter culture (1.2 mL) was added directly to the 1 L culture, and the cells were grown at room temperature until the OD_{600} reached 0.8. Protein expression was induced using 1 mM IPTG, and the cells were harvested by centrifugation for 24 h after induction. Expression of the recombinant GSAP was confirmed by Western blotting using a monoclonal anti-5X His mouse antibody (Cell Signaling Technology, Danvers, MA).

Purification of N- and C-Terminally His_6-Tagged GSAP. The harvested cells were weighed and lysed in 20 mL of lysis buffer [75 mM Tris, 300 mM NaCl, and 0.2 mM EDTA (pH 7.8)] per gram of cells. Also added were 5 mM magnesium acetate, 2 mg/mL lysozyme, 0.2 mg/mL DNase and RNase, and 50 μL of protease inhibitor per gram of cells. The suspension was tumbled for 90 min at room temperature. After being tumbled, cells were further disrupted by a 5 min probe sonication with a 50% duty cycle at approximately 57 W using a Misonix (Farmingdale, NY) sonicator. The lysate was centrifuged at 20000 rpm in a Beckman-Coulter (Indianapolis, IN) JA 25.5 rotor (approximately 48000*g*), and the pellet, which includes inclusion bodies, was retained. The inclusion bodies containing GSAP were solubilized in 20 mL of lysis buffer per original gram of cells using 3% (v/v) Empigen, a harsh detergent. This solution was tumbled at room temperature until a clear mixture was observed (approximately 4.5 h). The sample was then centrifuged to remove any remaining insoluble particulates. Ni-NTA resin (1.2 mL/g of cells) was equilibrated with buffer A [40 mM HEPES and 300 mM NaCl (pH 7.8)]. The supernatant was tumbled with the resin for 1 h at room temperature. The resin was loaded into a column and sequentially washed with buffer A containing 3% (v/v) Empigen and buffer A containing 30 mM imidazole and 1.5% (v/v) Empigen, to elute all non-His_{10}-tagged proteins from the resin. Empigen was then exchanged for the detergent *n*-dodecylphosphocholine (DPC) by re-equilibrating the column with 12 column volumes of 20 mM phosphate (pH 7.2) containing 0.5% (w/v) DPC. GSAP was eluted from the column with 250 mM imidazole containing 0.5% (w/v) DPC (pH 7.8). Purification was monitored by A_{280}. After purification, 2 mM DTT was added to the sample to reduce disulfide bonds.

The purification process was monitored by SDS−PAGE. Electrophoresis experiments were conducted using an Invitrogen (Grand Island, NY) Novex-Mini Gel system and NuPAGE 4 to 12% Bis-Tris polyacrylamide gels and MES running buffer.

Purification of the Thioredoxin-His_6-GSAP Fusion Protein. The purification of Trx-His_6-GSAP was mentioned but not described in the original paper[8] but is similar to the His_{10}-GSAP purification strategy described above (personal communication). Trx-His_6-GSAP-expressing cells were grown, harvested, and lysed as described above. After lysis, the supernatant was collected and bound to Ni-NTA resin equilibrated in 50 mM phosphate and 500 mM NaCl (pH 7.8). The slurry was tumbled for 1 h at room temperature. The resin was rinsed with 50 mM phosphate and 500 mM NaCl (pH 7.8) and then washed with 50 mM phosphate, 500 mM NaCl, and 40 mM imidazole (pH 7.8) to remove any remaining nonspecifically bound protein. Trx-His_6-GSAP was eluted from the column with 50 mM phosphate, 500 mM NaCl, and 300 mM imidazole (pH 7.8). The eluate was concentrated to a volume of <5 mL and filtered with a 0.2 μm filter. The sample was then subjected to size exclusion chromatography using a HiPrep Sephacryl S300 16/60 gel filtration column on an AKTAprime-plus fast performance liquid chromatography system eluted with 20 mM HEPES, 200 mM NaCl, and 1 mM EDTA (pH 8.0).

Circular Dichroism (CD) Spectroscopy. Protein samples were purified as described above and were exchanged into a 25 mM sodium phosphate buffer (pH 7.5) containing 0.5% DPC

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using a PD-10 desalting column (Bio-Rad). The sample and buffers were passed through a 0.2 μm filter before CD data were collected. Far-UV CD data were collected from 190 to 260 nm on a Jasco (Easton, MD) J-810 CD spectropolarimeter. Data from five scans were averaged together and blank-corrected.

Solution NMR Spectroscopy. For NMR spectroscopy, the pH of GSAP was adjusted to 7.5 and D_2O was added to a final concentration of 10%, followed by concentration through ultrafiltration using an Amicon Centrifugal Filter unit molecular mass cutoff of 10000 Da (Millipore, Billerica, MA). An HSQC spectrum was collected on a 600 MHz Bruker AVANCE III spectrometer at 298 K using TopSpin3 and a standard Bruker pulse sequence.

Titration of C99 by GSAP. Uniformly ^{15}N-labeled C99 with a His_6-containing purification tag at its C-terminus was expressed and purified as described by Beel et al.,[11] with a few minor variations. Cultures of *E. coli* with an expression vector encoding C-terminally His_6-tagged human C99 were grown at 37 °C in minimal medium with Cellgro MEM vitamins and induced at an OD_{600} of 0.8 using IPTG, at 18 °C overnight. Cells were lysed, and inclusion bodies were isolated and washed three times with lysis buffer followed by sonication and recentrifugation. Tagged C99 was then purified using Ni-NTA affinity chromatography in 0.05% LMPG micelles with 250 mM imidazole (pH 7.8). After purification, [U-^{15}N]C99 was buffer-exchanged and centrifugally concentrated in Amicon concentrators to a final condition of 0.6 mM, with 2.5% LMPG and 100 mM imidazole. The pH was adjusted to 7.5 using glacial acetic acid and ammonium hydroxide. Unlabeled GSAP was prepared as described above using 0.05% LMPG as the detergent for the final purification steps. The purified protein was buffer-exchanged to reduce the imidazole concentration to 100 mM and concentrated to 0.45 mM. The pH was adjusted to 7.5, and LMPG was added to a final concentration of 2.5%.

For titrations, NMR samples were prepared with 0.1 mM C99 in each sample and increasing GSAP:C99 molar ratios of up to 4:1, in 100 mM imidazole and 2.5 or 10% LMPG. $^1H-^{15}N$ TROSY spectra were collected at 298 K for each sample to determine the effect of GSAP on the chemical shifts of the peaks in the C99 spectrum.

Titration of Imatinib with GSAP. GSAP was prepared as described, except that the final buffers were made using D_2O. The sample was concentrated and passed over a PD-10 desalting column to remove all traces of imidazole. This sample contained 0.325 mM GSAP in 25 mM sodium phosphate in D_2O and 1% DPC (pH 7.5) and served as a stock solution for the titration. Starting with a solution of 339 mM imatinib mesylate in DMSO, a 1 mM stock solution of the drug in 1% (w/v) DPC in D_2O was prepared. The NMR samples were prepared with 50 μM imatinib, 25 mM imidazole, and increasing amounts of GSAP up to a 4-fold molar excess. The imatinib one-dimensional (1D) ^1H NMR peaks not obscured by detergent and protein were monitored for changes with increasing amounts of GSAP using a 600 MHz magnet at 298 K.

RESULTS

Expression and Purification of GSAP. The GSAP domain of PION[8] was cloned into pET vectors. Two constructs, one with an N-terminal (pET16) His_6 and one with a C-terminal (pET21) His_{10} purification tag, were cloned. Both constructs were overexpressed well in different strains of *E. coli* [BL21(DE3) and Rosetta(DE3)]. It was found that the N-terminally tagged construct was strongly expressed but was highly unstable and prone to aggregation. For this reason, our experiments were conducted using the C-terminally tagged construct, which behaved more favorably.

Following expression, cell lysis, and centrifugation, GSAP was located primarily in inclusion bodies (IB), despite being cultured the cells in minimal medium at room temperature, conditions sometimes found to promote folding of unstable or misfolding-prone recombinant proteins. Consequently, IB solubilization and protein refolding were necessary. Solubilization methods that were tested included dissolution of IB in 8 M urea and 0.2% sodium dodecyl sulfate (SDS), both together and separately, followed by removal of the denaturant under different buffer and pH conditions, from pH 5.5 to 7.8. Unfortunately, despite much effort, all refolding attempts resulted in precipitation of GSAP. For further details about refolding attempts and outcomes, see Table 1 of the Supporting Information.

Additional experiments were conducted in an effort to refold the protein, this time with GSAP immobilized by being bound to Ni-NTA resin through its His_{10} tag. Inclusion bodies were solubilized with 8 M urea and 0.2% SDS and incubated with Ni-NTA resin. The first on-column refolding test involved the stepwise removal of SDS and urea from the solution bathing the resin. After complete removal of the denaturant, an attempt was made to elute the protein from the column with 250 mM imidazole (pH 7.8). However, GSAP did not elute, indicating insolubility in a denaturant-free and detergent-free elution buffer. On the basis of the knowledge that GSAP can be solubilized using a harsh detergent (SDS), additional attempts were made to refold GSAP in the presence of a milder detergent.

Inclusion bodies were solubilized using the harsh zwitterionic detergent Empigen, and GSAP was then associated with the nickel resin. The detergent present in the solution that bathes the Ni-NTA-bound GSAP was then switched from Empigen to one of several detergents [DPC, lyso-myristoylphosphatidylglycerol (LMPG), or decyl maltoside (DM)], followed by attempted elution using 250 mM imidazole in that same detergent solution. It was found that GSAP could not be eluted in DM detergent micelle solutions but did elute when either DPC or LMPG solutions were used. Both of these detergents have previously been widely used as membrane mimetics in studies of membrane proteins.[12] Figure 1 shows an SDS–PAGE gel that documents protein purification. The elution fraction has only two bands, which have been confirmed by mass spectrometry to be the monomeric and dimeric forms of GSAP. The dimer band is likely due to the presence of the single cysteine at amino acid position 32, as this band is absent in the presence of a reducing agent. The total yield of the purified protein was approximately 15 mg/L of culture.

These results indicate that GSAP is highly prone to the formation of insoluble aggregates. Despite extensive testing of refolding conditions, we found the protein could be solubilized only in the presence of detergents or denaturing agents.

We next tested a fusion protein form of GSAP. In previous work,[8] recombinant GSAP was expressed as a fusion protein with thioredoxin, a widely used fusion partner for enhancing the solubility and stability of partner proteins.[13,14] We therefore constructed and tested a thioredoxin-His_6-GSAP-thioredoxin fusion protein. We found that the fusion protein also was expressed primarily in inclusion bodies. A small fraction that

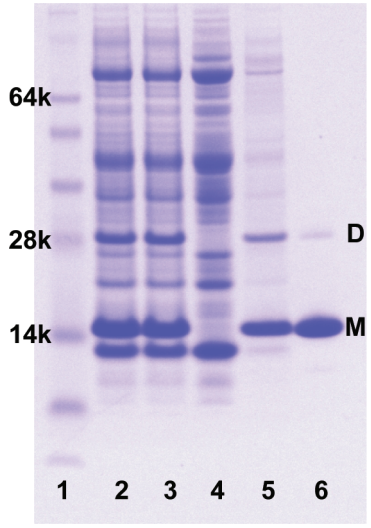

Figure 1. SDS–PAGE documentation of GSAP purification. A 4 to 12% polyacrylamide gel was stained with Coomassie R-250 brilliant blue: lane 1, SeeBlue Plus 2 protein molecular mass markers; lane 2, whole cell lysate solubilized with 4 M urea and SDS; lane 3, sonicated cell lysate further solubilized with 4 M urea and SDS; lane 4, cellular supernatant after centrifugation; lane 5, insoluble inclusion bodies solubilized with SDS; lane 6, purified protein fraction after elution with 250 mM imidazole and 0.5% DPC. All samples were first mixed with an SDS loading buffer prior to being loaded on the gel. M denotes monomeric GSAP and D dimeric GSAP, as confirmed by mass spectrometry.

was expressed in soluble form in the supernatant was associated with Ni-NTA resin but could not then be eluted from the column in the absence of a harsh detergent such as SDS. The amount of protein that was purified without detergent was negligible.

We also attempted to generate soluble GSAP without a fusion partner in the commercial competent cell line SoluBL21 (AMS Biotechnology, El Toro, CA). This cell line has been modified to enhance the solubility of difficult proteins and to allow for soluble expression where no soluble expression is seen in standard competent cell lines. When expressed in SoluBL21 cells, GSAP was initially soluble based on detection of a GSAP band on an SDS–PAGE gel in the supernatant of the cell lysate after centrifugation. However, after binding to the nickel resin followed by elution of all impurities in a low-concentration imidazole buffer, GSAP failed to elute from the nickel resin in the presence of 250 mM imidazole. Application of SDS to the resin released the protein. This suggests that while expression conditions can be found that initially produce a soluble form of GSAP, the protein is highly susceptible to aggregation. This result implies that a propensity of GSAP to aggregate is an intrinsic property of this protein.

Characterization of Solubilized GSAP. The properties of GSAP were examined in detergent-containing solutions in which the protein was soluble. Secondary structure predictions indicate that GSAP is largely α-helical with stretches of random coil or unstructured loops between helices. Near-UV CD spectroscopy in the 250–310 nm range [25 mM $NaPO_4$ (pH 7.5)] revealed a flat spectrum, providing no evidence of stable tertiary structure (data not shown). The far-UV CD spectrum collected under the same sample conditions shows a pattern consistent with mostly helical secondary structure (Figure 2). Analysis of this spectrum using the secondary structure prediction server K2D3 suggests that GSAP is largely (~92%) helical.[15]

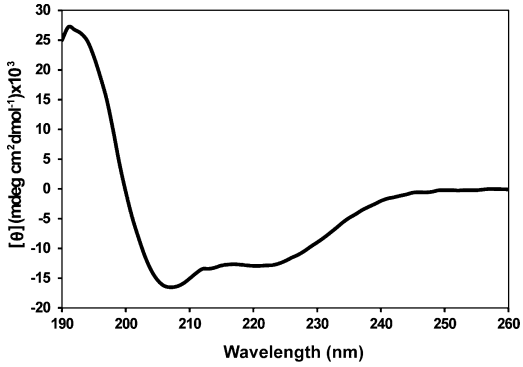

Figure 2. Estimation of secondary structure from far-UV CD spectroscopic data. This spectrum indicates that GSAP is largely α-helical with approximately 92% helicity based on analysis using the K2D3 secondary structure prediction server. Data represent an average of five scans.

Two-dimensional $^1H-^{15}N$ HSQC NMR spectra are routinely used to provide general insight into protein structure. All NMR spectra of GSAP-containing samples were collected at pH 7.5 because the protein precipitated when the pH was reduced to a neutral or acidic value. The HSQC spectrum of [^{15}N]GSAP in DPC micelles is shown in Figure 3 and is poorly dispersed,

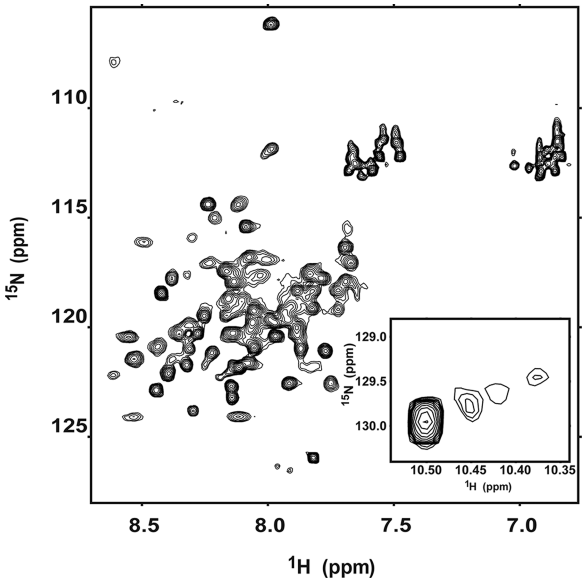

Figure 3. $^1H-^{15}N$ HSQC NMR spectrum (600 MHz) of GSAP. The sample contains ~300 μM uniformly ^{15}N-labeled GSAP in 0.5% DPC (pH 7.5), 2 mM DTT, and 10% D_2O at 298 K. The protein precipitated when the pH was reduced to neutral or acidic values. The inset highlights the tryptophan indole NH peaks from the single tryptophan in the protein.

showing only a fraction of the expected 126 backbone amide peaks. This spectrum is consistent with GSAP being largely α-helical but lacking well-defined tertiary structure, suggestive of a conformationally heterogeneous globular protein. The fact that there is only a single tryptophan in the protein but four peaks are observed in the indole side chain resonance region of the spectrum is consistent with the protein populating multiple

conformations that are only slowly exchanging on the NMR time scale.

GSAP has previously been shown to bind both the small molecule kinase inhibitor imatinib and the 99-residue transmembrane C-terminal domain of the amyloid precursor protein (C99), which serves as the substrate for γ-secretase cleavage to produce the amyloid-β polypeptides.[8] However, in neither case is it clear whether binary GSAP−imatinib or GASP−C99 complexes are formed, or whether they form complexes only in the presence of a tertiary partner such as γ-secretase. We therefore tested whether recombinant GSAP can form binary complexes with either imatinib or GSAP.

^{1}H−^{15}N HSQC NMR spectra of 150 μM isotopically labeled GSAP were collected in the absence and presence of a 1:1 molar ratio of imatinib. No shifts in GSAP resonances were seen upon addition of imatinib, as shown in Figure 4. In the

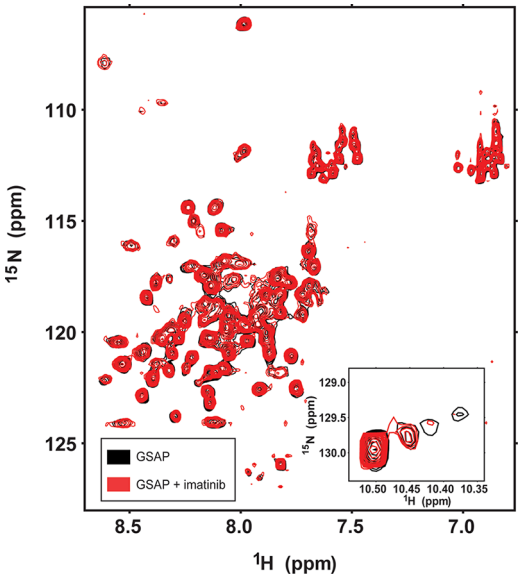

Figure 4. Attempt to detect interaction of GSAP with imatinib. An overlay of 600 MHz HSQC NMR spectra of [U-^{15}N]GSAP without imatinib (black) and GSAP in the presence of a 1:1 molar ratio of imatinib (red) is shown. These samples contained 0.15 mM GSAP in 0.5% DPC (pH 7.5), 2 mM DTT, and 10% D_2O at 298 K. The inset highlights the tryptophan indole NH peaks from the single tryptophan in the protein.

reverse experiment, 1D NMR spectra of 50 μM imatinib were recorded upon titration with increasing amounts of GSAP. Addition of GSAP to a 4-fold molar excess relative to the drug did not significantly affect the imatinib peaks (Figure 5). The results suggest that affinity between GSAP and imatinib under the tested conditions is weak or nonexistent.

To test for formation of a complex between C99 and GSAP, ^{1}H−^{15}N HSQC NMR was used to monitor the titration of uniformly ^{15}N-labeled C99 by increasing molar ratios of unlabeled GSAP. These experiments were initially conducted in 2.5% (w/v) LMPG micelles. Under these conditions, only modest and nonsaturable changes were seen in backbone amide ^{1}H and ^{15}N peak positions (Figure 6), consistent with nonspecific or weak interactions between these two proteins under the conditions used in this experiment. Indeed, while the GSAP interaction domain on C99 was proposed to be localized to residues 725−735 in the juxtamembrane cytosolic domain,[8] peaks from this domain were no more likely to undergo large

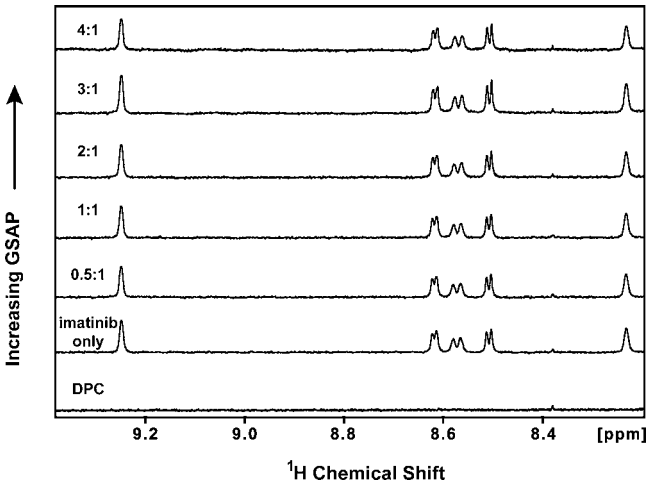

Figure 5. Titration of imatinib by GSAP, as monitored by 600 MHz 1D ^{1}H NMR. The aromatic regions of the 1D ^{1}H NMR spectra of 50 μM imatinib are shown as a function of increasing GSAP concentration: 0, 25, 50, 100, and 200 μM. Spectra were acquired in the presence of 1% DPC and 100% D_2O at pH 7.5 and 298 K and normalized to an internal standard. The listed ratios are the GSAP:imatinib molar ratios.

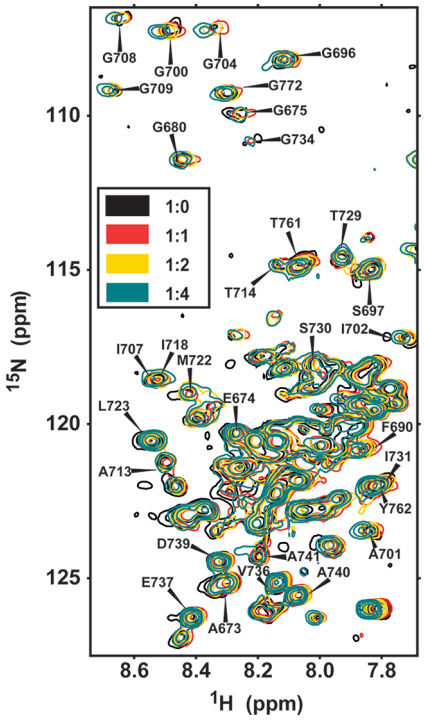

Figure 6. Titration of [U-^{15}N]C99 by unlabeled GSAP, as monitored by 600 MHz ^{1}H−^{15}N TROSY NMR. The samples contained 2.5% LMPG at pH 7.5 and 298 K. The listed ratios are the C99:GSAP molar ratios.

shifts in response to GSAP than peaks found in the transmembrane domain (residues 700−723) or from the extracellular domain of C99 (residues 672−699), the latter of which is located on the other side of the membrane from GSAP under physiological conditions. An additional titration was completed in which the same protein concentrations were used but the detergent (LMPG) concentration was increased to 10%. Under these conditions, little to no chemical shift changes were seen for C99 peaks upon titration by GSAP. The fact that

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the GSAP-induced changes in the spectrum seen at 2.5% LMPG in Figure 6 can be eliminated by increasing the micelle concentration (at a fixed protein level) suggests that GSAP has some affinity for the micelle surface that leads to nonspecific interaction between GSAP and C99 when both are confined to the same micelle, an interaction that can be minimized by simply adding excess (C99-free) micelles, to which GSAP will redistribute. These results indicate that any binding of GSAP to C99 in LMPG micelles is either nonspecific or very weak. These results do not, of course, rule out the possibility that GSAP and C99 do specifically and avidly interact, but only when both are bound to γ-secretase.

DISCUSSION

The notion that GSAP represents a protein that can be targeted by an already-approved drug to reduce the level of production of the amyloid-β polypeptides is extremely appealing. Accordingly, there is a compelling impetus to conduct biochemical and biophysical studies of the structure and interactions of this protein. Unfortunately, on the basis of the work presented here, working with GSAP is likely to be challenging. It appears to be insoluble under many conditions and seems to be conformationally heterogeneous under detergent micellar conditions in which it is soluble. These observations hold regardless of the nature of the protein construct, regardless of the *E. coli* expression strain, and regardless of the refolding methods and final solution composition. While we cannot rule out the possibility that a refolding method and/or solution conditions may ultimately be found in which GSAP is both soluble and well-folded, we were not able to identify any such conditions despite considerable effort.

Under conditions in which GSAP is solubilized by the presence of DPC micelles, it was seen to be a mostly α-helical protein but was conformationally heterogeneous. It was also observed that under micellar conditions GSAP does not undergo specific association with either imatinib or the C99 domain of the amyloid precursor protein. It does not appear that the conformationally heterogeneous form of GSAP can be induced to adopt stable tertiary structure by interaction with either of these potential binding partners.

Despite the failure in this work to observe formation of well-ordered tertiary structure by GSAP or formation of a complex with either imatinib or C99 titrations, our results are not definitively negative. We cannot rule out the possibility that either an unidentified refolding pathway or folding-favorable final solution conditions that we have not yet discovered do exist. In native mammalian cells, it is possible that chaperones might facilitate a folding outcome different from what we have observed working with recombinant protein. We also cannot rule out the possibility that GSAP is subject to an unidentified post-translational modification under mammalian cellular conditions that is required for the folding or solubility in detergent-free solutions. While GSAP was not seen to form binary complexes with either imatinib or C99, it may do so under mammalian cellular conditions, perhaps as a result of the formation of a ternary complex with an additional binding partner such as γ-secretase.

Our results should not be taken to imply a challenge of the data or interpretations regarding the GSAP protein as presented in previous work.[8,9] The previous studies were conducted primarily using cell-based methods involving model mammalian cell lines. However, for those considering the pursuit of biophysical studies of this protein, our results suggest that work with recombinant GSAP may prove to be difficult. We were unable to find conditions under which this protein is water-soluble to an appreciable degree unless detergent micelles were used to facilitate solubilization, presumably by stabilizing a hydrophobic surface on GSAP that otherwise drives aggregation. When solubilized, GSAP was found to be mostly helical, though it did not adopt a stable and homogeneous tertiary structure. Nevertheless, we cannot rule out the possibility that further exploration of expression, purification, and protein refolding methods may eventually lead to a form of purified GSAP that is soluble, folded, and competent to bind imatinib and/or C99.

ASSOCIATED CONTENT

Supporting Information

Two supporting tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

Aβ, amyloid-β peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; CD, circular dichroism; DM, *n*-decyl maltoside; DPC, *n*-dodecylphosphocholine; DTT, dithiothreitol; GSAP, γ-secretase activating protein; IPTG, isopropyl β-D-1-thiogalactopyranoside; LMPG, lyso-myristoylphosphatidylglycerol; Ni-NTA, Ni(II) complex with nitrilotriacetic acid-derivatized agarose beads; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PION, pigeon homologue protein; SDS, sodium dodecyl sulfate.

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