Research Article

Spectroscopic Characterization of Intermolecular Interaction of Amyloid β Promoted on GM1 Micelles

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Clusters of GM1 gangliosides act as platforms for conformational transition of monomeric, unstructured amyloid β ($A\beta$) to its toxic β -structured aggregates. We have previously shown that $A\beta$ (1–40) accommodated on the hydrophobic/hydrophilic interface of lyso-GM1 or GM1 micelles assumes α -helical structures under ganglioside-excess conditions. For better understanding of the mechanisms underlying the α -to- β conformational transition of $A\beta$ on GM1 clusters, we performed spectroscopic characterization of $A\beta$ (1–40) titrated with GM1. It was revealed that the thioflavin T- (ThT-) reactive β -structure is more populated in $A\beta$ (1–40) under conditions where the $A\beta$ (1–40) density on GM1 micelles is high. Under this circumstance, the C-terminal hydrophobic anchor Val³⁹-Val⁴⁰ shows two distinct conformational states that are reactive with ThT, while such $A\beta$ species were not generated by smaller lyso-GM1 micelles. These findings suggest that GM1 clusters promote specific $A\beta$ - $A\beta$ interactions through their C-termini coupled with formation of the ThT-reactive β -structure depending on sizes and curvatures of the clusters.

1. Introduction

Conformational transitions of unstructured proteins into β -structure-based oligomeric or amyloid states are crucial processes in the onset and development of a variety of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease [1, 2]. Amyloid β ($A\beta$), a major player in AD, is a 40- or 42-amino acid peptide cleaved from its precursor membrane protein by sequential actions of β - and γ -secretases and has a high propensity for toxic aggregation to form cross- β -fibrils [3, 4]. Accumulated evidence indicates that the GM1 ganglioside, a glycosphingolipid abundant in neuronal cell membranes, interacts with $A\beta$ and promotes its assembly, resulting in pathogenic amyloid formation [5–7]. For example, high-density GM1 clustering, which is exclusively observed in synaptosomes, is suggested to

accelerate $A\beta$ deposition [8]. *In vitro* experiments have indicated that the $A\beta$ -GM1 interaction depends on the clustering of GM1, and its carbohydrate moiety alone cannot induce conformational changes of $A\beta$ [15, 30, 31].

Furthermore, it has been suggested that each of the heredity variants of $A\beta$ reported thus far has its own specificities for gangliosides, which have been supposed to be associated with their ectopic deposition [9, 10]. Promotion of amyloid formation in membrane-bound states has also been reported for prion and α -synuclein [11, 12]. For example, prion protein has been reported to be localized in the membrane microdomains and caveolae enriched with ganglioside, which interacts with prion protein and thereby promotes its α -to- β structural conversion [13, 14]. Therefore, detailed conformational characterization of $A\beta$ interacting with the ganglioside clusters not only provides

structural information as cues for drug development in preventing and treating AD but also offers general insights into the mechanisms underlying the disease-associated amyloid formation facilitated in membrane environments.

In previous papers, we have reported nuclear magnetic resonance (NMR) studies of the interactions of A β (1–40) with ganglioside clusters using lyso-GM1 micelles (approximate molecular mass 60 kDa) as model systems [15, 16]. Our NMR data showed that A β (1–40) is accommodated on the hydrophobic/hydrophilic interface of the ganglioside cluster exhibiting an α -helical conformation under ganglioside-excess conditions. In this state, A β (1–40) shows an up-and-down topological mode in which the two α -helices at segments His¹⁴-Val²⁴ and Ile³¹-Val³⁶ and the C-terminal Val³⁹-Val⁴⁰ dipeptide segment are in contact with the hydrophobic interior of the micelles, whereas the remaining regions are exposed to the aqueous environment. A similar tendency of A β (1–40) has been observed using excess amounts of GM1, which forms micelles with an approximate molecular mass of 140 kDa [15, 17]. These findings indicate that ganglioside clusters offer unique platforms at their hydrophobic/hydrophilic interfaces for binding coupled with α -helix formation of A β molecules.

To gain further insights into the underlying mechanisms of the amyloid formation of A β , it is necessary to characterize the conformational transition from α -helices to β -structures on the ganglioside clusters. On the basis of the circular dichroism (CD) data, Kakio et al. demonstrated that A β /GM1 ratios influence the secondary structure of A β (1–40) on the raft-like lipid bilayers composed of GM1, cholesterol, and sphingomyelin [18, 19]. Namely, A β adopts an α -helical structure at lower A β /GM1 ratios (≤ 0.025), while it assumes a β -sheet-rich structure at higher ratios (≥ 0.05). Although more detailed structural information on A β bound to the GM1 cluster is highly desirable, the small unilamellar vesicles used for the CD measurements are still too large to investigate with solution NMR techniques.

In the present study, we attempt to characterize conformational states of A β (1–40) in the presence of varying amounts of GM1 aqueous micelles using stable-isotope-assisted NMR spectroscopy in conjunction with synchrotron-radiation vacuum-ultraviolet CD (VUVCD) spectroscopy. We found that GM1 micelles also induce distinct secondary structures of A β (1–40) depending on the A β /GM1 ratios. On the basis of the spectroscopic data, we will discuss A β behaviours on the ganglioside clusters from a structural point of view.

2. Materials and Methods

2.1. Preparation of A β (1–40). Recombinant A β (1–40) was expressed and purified as a ubiquitin extension. The plasmid vector encoding A β (1–40) was constructed and cloned as a fusion protein with hexahistidine-tagged ubiquitin (His₆-Ub) using the pET28a(+) vector (Novagene), subsequently transformed into *Escherichia coli* strain BL21-CodonPlus (Stratagene) [15]. Transformed bacteria were grown at 37°C in LB media containing 15 μ g/mL of kanamycin. For the production of isotopically labelled A β (1–40) protein,

cells were grown in M9 minimal media containing [¹⁵N] NH₄Cl (1 g/L) and/or [U-¹³C₆] glucose (2 g/L). Protein expression was induced by adding 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the absorbance reached 0.8 at 600 nm. After 4 hours, cells were harvested and then suspended into buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, subsequently disrupted by sonication. After centrifugation, the pellet was dissolved in buffer A containing 8 M urea. His₆-Ub-A β (1–40) was purified by a Ni²⁺-nitrilotriacetic acid affinity column (GE Healthcare). Recombinant glutathione S-transferase- (GST-) tagged yeast ubiquitin hydrolase-1 (YUH-1) was grown until the absorbance reached 0.8 at 600 nm and then induced to express by IPTG. Cell pellets were dissolved in buffer B (50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.5) and disrupted by sonication. GST-YUH-1 was purified by a glutathione affinity column (GE Healthcare). A β (1–40) protein was enzymatically cleaved from His₆-Ub by incubation with GST-YUH-1 for 1 h at 37°C at a molar ratio of His₆-Ub-A β (1–40): GST-YUH-1 = 10 : 1. The cleaved A β (1–40) was purified by reverse-phase chromatography using an octadecylsilane column (TSKgel ODS-80T_M, TOSOH) with a linear gradient of acetonitrile. The fraction containing A β (1–40) was collected and lyophilized.

Synthetic A β (1–40) labelled with ¹⁵N selectively at Val³⁹ or Val⁴⁰ was purchased from AnyGen Co. Both of recombinant and synthetic A β (1–40) proteins were dissolved at an approximate concentration of 2 mM in 0.1% (v/v) ammonia solution then collected and stored in aliquots at –80°C until use.

2.2. Preparation of Micelles. Powdered lyso-GM1 and GM1 were purchased from Takara Bio Inc. and Sigma-Aldrich, respectively. These gangliosides were dissolved in methanol. Subsequently, the solvent was removed by evaporation. The residual ganglioside was suspended at a concentration of 12 mM in 10 mM potassium phosphate buffer (pH 7.2) and then mixed by vortexing. Micelle sizes were determined by dynamic light scattering using a DynaPro Titan (Wyatt technology).

2.3. Thioflavin T (ThT) Assay. A β (1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer (pH 7.2) in the absence or presence of 0.4–9 mM GM1 or lyso-GM1. The samples were kept on ice before measurements. 980 μ L of 5 μ M ThT (Sigma) solution in 50 mM glycine-NaOH buffer (pH 8.5) was added to an aliquot of 20 μ L of each sample. Fluorescence was measured immediately after mixing at the excitation and emission wavelengths of 446 and 490 nm, respectively, [20] using spectrofluorophotometer (Hitachi F-4500) at 37°C.

2.4. VUVCD Measurements. A β (1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer (pH 7.2). The CD spectra of A β (1–40) in the presence or absence of GM1 were measured from 265 to 175 nm under a high vacuum (10^{–4} Pa) at 37°C using the VUVCD spectrophotometer constructed at beamline

15 (0.7 GeV) of the Hiroshima Synchrotron Radiation Center (HiSOR). Details of the spectrophotometer and optical cell were described previously [21, 22]. The path length of the CaF₂ cell was adjusted with a Teflon spacer to 50 μ m or 100 μ m for measurements. The VUVCD spectra were recorded with a 1.0-mm slit, a 16-s time constant, a 4-nm min⁻¹ scan speed, and nine accumulations. The molar ellipticities of A β (1–40) were calculated with the average residue weight of 107.5. The secondary structure contents of A β (1–40) were analysed using the modified SELCON3 program [23] and the VUVCD spectra down to 160 nm for 31 reference proteins with known X-ray structures [24, 25]. The secondary structures of these proteins in crystal form were assigned into four classes (α -helices, β -strands, turns, and unordered structures) using the DSSP program [26] based on the hydrogen bonds between adjacent amide groups. In this analysis, the ₃₁₀-helix was classified as an unordered structure. The root-mean-square deviation (δ) and the Pearson correlation coefficient (r) between the X-ray and VUVCD estimates of the secondary structure contents of the reference proteins were 0.058 and 0.85, respectively, confirming the high accuracy of the VUVCD estimation [27].

2.5. NMR Measurements. NMR spectral measurements were made on a Bruker DMX-500 spectrometer equipped with a cryogenic probe as well as a Bruker AVANCE III-400 spectrometer. The probe temperature was set to 37°C. Isotopically labelled A β (1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer (pH 7.2) containing 10% (v/v) ²H₂O in the presence or absence of GM1. For ¹H-¹⁵N heteronuclear single-quantum correlation (HSQC) measurements, the spectra were recorded using A β (1–40) labelled with ¹⁵N uniformly or selectively at the amide group of Val³⁹ or Val⁴⁰ at a ¹H observation frequency of 500 MHz with 128 (t_1) $\times$ 1024 (t_2) complex points and 256 scans per t_1 increment. The spectral width was 1720 Hz for the ¹⁵N dimension and 6000 Hz for the ¹H dimension.

One-dimensional carbonyl ¹³C spectra were recorded using uniformly ¹³C- and ¹⁵N-labelled A β (1–40) at a ¹H observation frequency of 400 MHz with a spectral width of 22,000 Hz. In these experiments, 32,768 data points for acquisition and 16,384 scans were acquired. NMR spectra were processed and analysed with the program nmrPipe/Sparky.

3. Results

3.1. ThT Fluorescence Enhancement. We examined whether ThT fluorescence is enhanced by A β (1–40) in the presence of varying concentrations of GM1 or lyso-GM1. As shown in Figure 1, GM1 exhibited a bell-shaped dependence on A β /GM1 ratios regarding ThT fluorescence enhancement, while lyso-GM1 showed virtually no enhancement. Maximum enhancement was observed at a 1:15 molar ratio of A β (1–40) to GM1. The dynamic light scattering data confirmed that the GM1 and lyso-GM1 micelles exhibited an approximate hydrodynamic radius of 6 nm and 4 nm, respectively, irrespective of the A β /ganglioside ratios. The

FIGURE 1: ThT fluorescence enhancement by A β (1–40) in the presence of varying concentrations of GM1 (open circle) or lyso-GM1 (closed circle). Each intensity value indicates the average of four values $\pm$ S.D.

FIGURE 2: VUVCD spectra of 0.2 mM A β (1–40) in the absence or presence of GM1. A β /GM1 molar ratios were 1:0 (black), 1:15 (blue), and 1:30 (red).

observed fluorescence intensity remained almost constant up to 12 h. These data indicated that GM1 micelles at appropriate A β /GM1 ratios promote some A β –A β interaction with formation of their β -sheet-like conformation, which, however, does not result in irreversible fibril formation.

3.2. Secondary Structure Transition. We characterized the conformational transition of A β depending on A β /GM1 ratios by CD measurements. The short-wavelength limit of CD spectroscopy can be successfully extended using synchrotron radiation as a high-flux source of photons, which yields much more accurate data than those obtained with a conventional CD spectrophotometer [28, 29]. The spectral data indicated that A β (1–40) undergoes conformational transitions depending on GM1 to A β (1–40) ratios (Figure 2).

FIGURE 3: Carbonyl ^{13}C spectra of uniformly ^{13}C -labelled $\text{A}\beta(1-40)$. Spectral data were obtained using 0.2 mM $\text{A}\beta(1-40)$ titrated with GM1 micelles at $\text{A}\beta/\text{GM1}$ molar ratios of (a) 1:0, (b) 1:15, and (c) 1:30. In (b), the spectra measured in the presence of ThT are displayed at $\text{A}\beta/\text{ThT}$ molar ratios of 1:0 (black), 1:1 (red), and 1:2 (blue).

TABLE 1: Secondary structure contents (%) of $\text{A}\beta(1-40)$ from VUVCD spectra obtained in the presence of varying concentrations of GM1.

$\text{A}\beta:\text{GM1}$	α -Helix	β -Strand	Turn	Unordered structure
1:0	15.9	17.8	26.3	39.0
1:15	23.6	23.6	21.6	29.3
1:30	40.0	18.3	14.5	27.9

The secondary structure contents of $\text{A}\beta(1-40)$ at $\text{A}\beta/\text{GM1}$ molar ratios of 1:0, 1:15, and 1:30 were estimated on the basis of the spectral data (Table 1). The α -helix content of $\text{A}\beta(1-40)$ in the presence of GM1 at an $\text{A}\beta/\text{GM1}$ molar ratio of 1:30 was calculated to be 40.0%, which is consistent with our previous estimation based on the backbone chemical shift data of lyso-GM1 [15], thus confirming close similarity of the binding modes of $\text{A}\beta(1-40)$ between GM1 and lyso-GM1 micelles. At an $\text{A}\beta/\text{GM1}$ molar ratio of 1:15, where the maximum ThT fluorescence enhancement was observed, the CD data consistently indicated a significantly increased content of β -strands.

The conformation of $\text{A}\beta(1-40)$ in the presence of varying amounts of GM1 micelles was further characterized by ^{13}C NMR spectroscopy. The carbonyl ^{13}C NMR spectral data of uniformly ^{13}C -labelled $\text{A}\beta(1-40)$ indicated that the peaks shifted upfield, roughly corresponding to β -structures, are more populated at an $\text{A}\beta/\text{GM1}$ molar ratio of 1:15 in comparison with the GM1-excess conditions (Figure 3). Intriguingly, intensities of these peaks were selectively reduced upon the addition of ThT. These NMR data are again

consistent with the VUVCD data as well as the results of the ThT assay.

3.3. Local Structure of the C-Terminus of $\text{A}\beta(1-40)$. To provide more detailed information on the conformational transition of $\text{A}\beta(1-40)$ on GM1 micelles, we observed ^1H - ^{15}N HSQC spectral changes of $\text{A}\beta(1-40)$ upon titration with GM1. Interestingly, at an $\text{A}\beta/\text{GM1}$ molar ratio of 1:15, $\text{A}\beta(1-40)$ exhibited HSQC peaks that were not observed in the spectra of free or fully micelle-bound forms (Supplementary Figure 1). By using site-specifically ^{15}N -labelled $\text{A}\beta$, these extra peaks were assigned to Val³⁹ and Val⁴⁰ (Figure 4 and Supplementary Figure 1 available online at doi:10.4061/2011/925073). Namely, the amide groups of these C-terminal residues of the micelle-bound $\text{A}\beta$ species show double HSQC peaks under the condition where $\text{A}\beta/\text{GM1}$ ratio is relatively high. More interestingly, these double peaks were perturbed upon the addition of ThT, while the corresponding peaks originating from the free and fully micelle-bound forms showed little or no change (Figure 4). On the other hand, many of the ^1H - ^{15}N HSQC peaks from $\text{A}\beta(1-40)$, including Val³⁹ and Val⁴⁰, were not observed at an $\text{A}\beta/\text{lyso-GM1}$ molar ratio of 1:15 due to intermediate chemical exchange between free and micelle-bound states of $\text{A}\beta(1-40)$ (data not shown).

4. Discussion

Accumulating evidence, including our previous reports, indicates that the interaction of $\text{A}\beta$ with GM1 involves multiple steps including the initial encounter complex formation and the accommodating process on the hydrophilic/hydrophobic interface of the ganglioside clusters [15–17, 30]. NMR spectral data of $\text{A}\beta(1-40)$ titrated with GM1 micelles under $\text{A}\beta$ -excess conditions indicated that they form a weak complex presumably through an interaction between the N-terminal segment of $\text{A}\beta(1-40)$ and the outer carbohydrate branch of GM1 [15, 30]. Thus, it is conceivable that the outer-branch structures of the carbohydrate moieties of gangliosides influence the association phase of the interaction and thereby determine the ganglioside specificities of $\text{A}\beta$. Nongangliosidic micelles and vesicles are barely or not capable of trapping $\text{A}\beta(1-40)$ effectively [15, 18, 31, 32]. On the other hand, the α -helical conformation of $\text{A}\beta(1-40)$ accommodated on sugar-lipid interface of the GM1 and lyso-GM1 micelles have been characterized by NMR under ganglioside-excess conditions ($\text{A}\beta/\text{ganglioside}$ molar ratio of 1:30) [15]. Because the structure of the inner part is common among the gangliosides, non-GM1 ganglioside, for example, GM2, can accommodate $\text{A}\beta$ and induce its α -helical conformation [16]. Thus, the spectroscopic characterization of the interactions of $\text{A}\beta$ with gangliosidic micelles has so far been performed only under the extreme conditions of the $\text{A}\beta/\text{ganglioside}$ ratios. The present study attempts to bridge the gap in our understanding of $\text{A}\beta$ behavior on GM1 micelles by carrying out spectroscopic analyses of $\text{A}\beta$ in the presence of varying amounts of GM1 micelles.

FIGURE 4: ^1H - ^{15}N HSQC peak originating from Val³⁹ (upper) and Val⁴⁰ (lower) of $\text{A}\beta(1-40)$ in the presence or absence of GM1 micelles and ThT. Site specifically ^{15}N -labelled $\text{A}\beta(1-40)$ proteins (0.2 mM each) were titrated with GM1 at $\text{A}\beta/\text{GM1}$ molar ratios of 1:0 (a, d), 1:15 (b, e), and 1:30 (c, f). The spectra measured in the absence (black) and presence (red) of 0.4 mM ThT are overlaid. The peak indicated by asterisk originated from GM1.

The present data all indicated that β -structure is more populated in micelle-bound $\text{A}\beta(1-40)$ under the condition where the $\text{A}\beta/\text{GM1}$ ratio is higher. It is intriguing that the increased β -structure is reactive with ThT. Although the binding mode of ThT to amyloid fibrils has yet to be fully elucidated, it has been suggested that ThT is more likely to bind perpendicularly to parallel β -strands in a β -sheet [33–35]. In addition, recently reported solid-state NMR data indicate that a ThT-reactive, neurotoxic amyloid intermediate of $\text{A}\beta(1-40)$ is composed of parallel β -structures [36]. These data suggest that formation of parallel β -strands is the minimum prerequisite for ThT fluorescence enhancement. With this in mind, the bell-shape dependence of ThT fluorescence enhancement (Figure 1) can be interpreted as

follows. At an extremely low concentration of GM1, most of $\text{A}\beta(1-40)$ exists as a free form, which is an unstructured monomer and therefore is not reactive with ThT. Fraction of the micelle-bound form of $\text{A}\beta(1-40)$ increases with increase of the GM1 amounts. To some extent, the micelles promote intermolecular interaction of $\text{A}\beta(1-40)$, giving rise to the ThT-reactive $\text{A}\beta(1-40)$ species. Under GM1-excess conditions, however, $\text{A}\beta(1-40)$ molecules are presumably relatively isolated from one another and therefore are not capable of forming an intermolecular β -structure. The $\text{A}\beta/\text{GM1}$ molar ratio, where the maximum enhancement was observed, was 1:15, which corresponds to average number of $\text{A}\beta/\text{micelle}$ of 11.2 with the assumption of the micellar GM1 aggregation number of 168 ± 4 [37]. Thus, the $\text{A}\beta$ density on

GM1 micelles is a crucial factor determining the occurrence of the ThT-reactive A β species.

Under the circumstance where the A β (1–40) density on GM1 micelles is high, the C-terminal dipeptide of A β (1–40) shows, at least, two distinct conformational states that are reactive with ThT. In a previous paper, we demonstrated that the C-terminal Val³⁹-Val⁴⁰ dipeptide is inserted into the hydrophobic interior of the gangliosidic micelles [15]. This C-terminal segment is involved in the parallel β -structure in the amyloid fibril and intermediate [36, 38]. On the basis of these data, we suggest that GM1 clusters promote intermolecular A β -A β interactions coupled with the conformational transition of their C-terminal hydrophobic anchors into the ThT-reactive parallel β -structure, in which the local chemical environments of the C-terminal segments are different in different β -strands. This may account for the multiple HSQC peaks originating from the C-terminal segments (Figure 4).

It has been reported that A β exhibits ThT-reactive β -sheet-rich aggregates in the presence of sodium dodecyl sulfate (SDS) at submicellar concentrations [39, 40]. Under these conditions, all the amide peaks of A β (1–40) disappeared from the ¹H-¹⁵N HSQC spectrum because of the formation of large aggregates, except for those from the C-terminal residues that should still be mobile in this assembly state. On the basis of the NMR data obtained using paramagnetic probes, the C-terminal segment of A β (1–40) bound to SDS micelles has shown to be exposed to aqueous environment, exhibiting higher mobility [41]. Taking into account these data in conjunction with our present data, we suggest that different β -like structures of A β (1–40) are induced by GM1 aqueous micelles and submicellar concentrations of SDS.

Lyso-GM1 micelles could not induce the formation of the ThT-reactive β -structure of A β (1–40) although the micelle-interacting modes of A β (1–40) are almost identical between GM1 and lyso-GM1 micelles under ganglioside-excess conditions [15]. By inspection of the dynamic light scattering data on an assumption of their globular shapes, the diameters of GM1 and lyso-GM1 micelles have been estimated as 12 nm and 8 nm, respectively. It is plausible that the sizes and curvatures of the gangliosidic micelles are determining factors for the number of A β molecules that can be accommodated on their hydrophilic/hydrophobic interface and the occurrence of A β -A β interactions coupled with ThT-reactive β -structure formation. Indeed, GM1 clusters with flatter curvature such as GM1-containing unilamellar vesicles induce enhanced A β fibrillogenesis [5] in comparison with GM1 micelles. Lipid composition can also be a determining factor for assembly states of GM1 molecules and their interaction with A β . Most importantly, there is growing evidence that cholesterol and sphingomyelin contribute to GM1 assembly and thereby influence A β deposition promoted by its cluster [8, 18, 42, 43]. Elucidation of the structural basis of these molecular events is an important subject for the forthcoming stage of the research.

In conclusion, in the present study, we firstly identified and characterized the ThT-reactive β -structure of A β (1–40) promoted on GM1 micelles. Our findings offer struc-

tural insights into the mechanisms underlying the α -to- β conformational transition of A β on GM1 clusters, which is associated with the nucleation process in the A β aggregation.

Abbreviations

A β :	Amyloid β
AD:	Alzheimer's disease
CD:	Circular dichroism
GST:	Glutathione S-transferase
His ₆ -Ub:	Hexahistidine-tagged ubiquitin
HSQC:	Heteronuclear single-quantum correlation
IPTG:	Isopropyl- β -D-thiogalactopyranoside
NMR:	Nuclear magnetic resonance
SDS:	Sodium dodecyl sulfate
ThT:	Thioflavin T
VUV:	Vacuum-ultraviolet
YUH-1:	Yeast ubiquitin hydrolase-1.

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