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Sumoylation of amyloid precursor protein negatively regulates A β aggregate levels

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Abstract

The proteolytic processing of amyloid precursor protein (APP) to produce A β peptides is thought to play an important role in the mechanism of Alzheimer's Disease. Here we show that lysines 587 and 595 of APP, which are immediately adjacent to the site of β -secretase cleavage, are covalently modified by SUMO proteins in vivo. Sumoylation of these lysine residues is associated with decreased levels of A β aggregates. Further, overexpression of the SUMO E2 enzyme ubc9 along with SUMO-1 results in decreased levels of A β aggregates in cells transfected with the familial Alzheimer's disease-associated V642F mutant APP, indicating the potential of up-regulating activity of the cellular sumoylation machinery as an approach against Alzheimer's Disease. The results also provide the first demonstration that the SUMO E2 enzyme (ubc9) is present within the endoplasmic reticulum, indicating how APP, and perhaps other proteins that enter this compartment, can be sumoylated.

Keywords

APP; SUMO-1; SUMO-2; ubc9

Introduction

Alzheimer's Disease is a debilitating condition that impairs cognitive function and is the most common aging-related human neurodegenerative disease [1–3]. It is widely believed that amyloid- β (A β) protein produced by processing of the amyloid precursor protein (APP) via the amyloidogenic proteolytic pathway is a primary causative factor in this disease.

Covalent attachment of Small Ubiquitin-like Modifier (SUMO) proteins to lysine residues in target proteins, or sumoylation, is an important regulator of protein functional properties [4–6]. SUMO proteins are covalently attached to target lysine residues by the SUMO E2 enzyme, ubc9, and these modified lysines are typically found within the consensus sequence Ψ KXE/D (Ψ represents hydrophobic amino acids) [7–10]. Cells express three major SUMO paralogs, SUMO-1, SUMO-2, and SUMO-3, with SUMO-2 and SUMO-3 being much more similar to each other than to SUMO-1 [4–6].

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Using an in vitro translation expression cloning strategy, in which candidate sumoylation substrate proteins were identified by assaying successive subdivisions of cDNA pools with in vitro sumoylation reactions, a previous study identified APP as a potential sumoylation substrate [11]. The goals of the experiments in this present study were to determine whether any lysine residue(s) within APP are sumoylated in the protein as expressed in cells, and if so, what role this modification plays in modulating the functional properties of this protein, including its proteolytic processing.

Materials and methods

Cell culture and plasmids

HeLa cells were cultured in DMEM medium (Cellgro) with 10% FBS and 1x antibiotic-antimycotic (Gibco, 100x) in 5% CO₂. Transfection was performed using Effectene reagent (Qiagen), following the manufacturer's protocol. The 6xHis-APP plasmid was constructed from pCITE-4a (+)-APP₆₉₅ (Dr. Iliya Lefterov). Mutagenesis PCR was performed to generate pCITE-APP K587R, K595R, K587/595R, and V642F mutants (QuikChange method, Stratagene). The pCITE-APP wildtype and mutants were then digested with *Bam*HI and *Not*I, and the APP fragments ligated into pAG3-His-APPΔNL plasmid [12] which was also digested with *Bam*HI and *Not*I, thereby swapping the Aβ-containing and flanking regions of the plasmids (thus, Swedish mutation is not present in final 6xHis-APP constructs). HA-SUMO-1 and HA-SUMO-2 were expressed using pcDNA3-HA-SUMO-1 and pcDNA3-HA-SUMO-2 plasmids (Dr. Kim Orth), and ubc9 expressed using a pcDNA3-ubc9 construct (Dr. Moshe Sadofsky).

His-tag pull-down of transfected proteins

HeLa cells were transfected with 6xHis-APP wildtype or mutant APP (V642F, K587R, K595R, or K587,595R) constructs along with HA-SUMO-1 or HA-SUMO-2 expression plasmids. At 48 hours after transfection, the cells were collected and re-suspended in 500μl pQE buffer (20mM Hepes (pH 7.4), 300mM NaCl, 2mM β-mercaptoethanol), with 1x protease inhibitor cocktail (Roche), 1mM PMSF, and 20mM N-ethylmaleimide added fresh. Cell lysis was performed by sonication 3 times at 20 kHz, followed by incubation on ice for 20 min. After centrifugation at 10,000 rpm, 4°C for 10 minutes, 80μl of the cell lysate was taken for analysis of Aβ and APP protein levels (20μl for each). 150μl of 50% Ni-NTA agarose slurry (Qiagen) was washed 3 times with PBS and then added to the cell lysate. After incubation at 4°C for 1hr, beads were washed sequentially with pQE buffers containing 5mM, 25mM, and 50mM imidazole (each wash twice). Then, 50μl pQE buffer with 250mM imidazole was added to the beads and protein eluted by shaking at RT for 30 min. The eluate was analyzed by anti-HA Western blot as described below.

Western blot antibodies

Antibodies used were: anti-Aβ(1–17) mouse antibody (Signet Labs), anti-APP C-terminal rabbit antibody (Calbiochem), anti-HA mouse antibody (gift of Dr. Doug Andres), anti-ubc9 and anti-lamin A mouse antibodies (both from BD Transduction Labs), and anti-calnexin rabbit antibody (Calbiochem).

Immunofluorescence microscopy

This was performed as previously described [13] using 1:100 dilutions of the anti-calnexin and anti-ubc9 antibodies.

Subcellular fractionation

HeLa cells were collected, and the cell pellets re-suspended in 500 μ l Buffer A (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 250 mM sucrose) with 1x protease inhibitor cocktail (Roche) added fresh. The cells were disrupted by dounce homogenization (30 strokes), followed by incubation on ice for 20 minutes. The cell lysate was then centrifuged at 1,000g, 4°C for 10 minutes to pellet nuclei. The supernatant was centrifuged at 10,000 $\times$ g, 4°C for 10 minutes to pellet mitochondria and intact Golgi. After this centrifugation, the supernatant was centrifuged at 20,000 $\times$ g, 4°C for 10 minutes to pellet large dense ER vesicles. The nuclei fraction and membrane fraction containing large dense vesicles were washed with Buffer A, and re-suspended in 10 pellet volumes of RIPA buffer (150mM NaCl, 50mM Tris-HCl (pH 7.4), 1% NP-40, 0.2% SDS, 0.25% sodium deoxycholate, 1mM EDTA, 1x protease inhibitor cocktail). The suspension was then passed through a 23 gauge needle 20 times, and incubated on ice for 20 minutes. After centrifugation at 10,000 rpm, 4°C for 10 min., the supernatant was analyzed by Western blot using antibodies that detect ubc9, lamin A, and calnexin. Sealed, ER-enriched, myelin-depleted calf brain microsomes were isolated as previously described [14], except that 10mM HEPES-NaOH (pH 7.4) was used instead of 0.1M Tris-HCl (pH 7.4). The intactness of these calf-brain microsomal vesicles was determined by measuring the latency of the processing, deoxynojirimycin-sensitive glucosidase I/II activities using [³H]Glc₁₋₃ Man₉GlcNAc₂ as substrate in the presence and absence of Triton X-100 (2mg/ml) as previously described [15].

Sealed microsome trypsin digestion assay

The calf-brain microsomal vesicles were incubated at 37°C for 30 minutes with trypsin (trypsin:total protein (w/w) = 1:20) in the absence or presence of 1% Triton X-100 (adapted from [15]), and then subjected to Western blot assay using anti-ubc9 antibodies.

Results

As shown in Fig. 1A, analysis of the amino acid sequence of APP revealed two matches to the sumoylation consensus sequence Ψ KXE/D surrounding lysines 587 and 595 of this protein (numbered according to the APP₆₉₅ isoform). These two lysine residues are immediately N-terminal to the β -secretase cleavage site. To test whether APP is modified by SUMO-1 or SUMO-2, and if so, whether the modification occurs at lysines 587 and/or 595, HeLa cells were transfected with expression plasmids encoding 6xHis-fusion constructs of wildtype APP and APP in which lysines 587 or 595, or both, were changed to non-sumoylatable arginines (K587R, K595R, and K587R/K595R), along with constructs encoding HA-SUMO-1 or HA-SUMO-2. The 6xHis tag in these APP constructs is located immediately C-terminal to the signal peptide, thereby allowing this 6xHis motif to remain after signal peptide cleavage and be used to affinity purify the transfected APP proteins using Ni-NTA beads [12]. Thus, extracts of the transfected cells were subjected to Ni-NTA affinity chromatography to pull down the transfected 6xHis-tagged wildtype and lysine mutant APP proteins, followed by anti-HA Western blot assay to detect potential sumoylated forms of the proteins. The results suggest that the wildtype APP protein is sumoylated by both SUMO-1 and SUMO-2 as evidenced by the detection of a number of distinct bands by the anti-HA antibody (Fig. 1B, top panels) that are significantly larger than the APP protein detected by an antibody that detects full-length APP (Figure 1B, bottom panels). The relatively large sizes of some of the sumoylated APP forms may reflect the presence of SUMO chains on the proteins [16], while the most rapidly migrating bands could possibly represent SUMO-modified proteolytic fragments of APP. The results also indicate that the intensity of sumoylated bands is slightly decreased for both the K587R and K595R APP mutants compared to wildtype APP, and completely abolished in the K587R/K595R double mutant APP protein. These results suggest that lysines 587 and 595 of APP are sites of sumoylation.

As shown above in Fig. 1A, the lysine 587 and 595 sumoylation sites in APP are only 1 and 9 residues, respectively, away from the β -secretase cleavage site. Based on this proximity, we hypothesized that sumoylation at these lysine residues could regulate cleavage at this site, thereby potentially modulating levels of A β . To test this hypothesis, we used an antibody against the A β peptide to perform Western blot assays of extracts of cells transfected with the wildtype or lysine mutant (both single and double mutants) APP constructs and HA-SUMO-1/HA-SUMO-2 constructs in order to examine levels of A β aggregates in the extracts [17–19]. The results indicate that, compared to the extracts of cells transfected with the wildtype APP protein, extracts of cells expressing the K587R and K595R single sumoylation site mutant APP constructs both exhibit higher levels of A β aggregates, which are further increased in those of cells expressing the K587R/K595R double mutant APP construct (Fig. 1B, middle panels). These results suggest that sumoylation of APP at lysines 587 and 595 acts to negatively regulate levels of A β aggregates.

Next, to test whether endogenous APP in brain is sumoylated, we subjected extracts of mouse brain to immunoprecipitation using an antibody against the C-terminal region of APP, followed by Western blot analysis of the immunoprecipitates using anti-SUMO-1 or anti-SUMO-2 antibodies. The results, shown in Fig. 2, indicate that brain APP is modified by SUMO-1 and SUMO-2.

Based on the results in Fig. 1 above indicating that blocking sumoylation of APP results in elevated levels of A β peptide aggregates, we hypothesized that up-regulating cellular sumoylation could represent a means for reducing A β levels. To test this hypothesis, we determined the effect of up-regulating expression of ubc9, the SUMO E2 enzyme, on levels of A β aggregates in cells transfected with an APP construct containing the familial Alzheimer's disease-associated V642F mutation that results in elevated levels of A β aggregates [20,21]. Thus, in this experiment cells were transfected with a 6xHis-APP (V642F) construct and the HA-SUMO-1 construct, and either empty pcDNA3 plasmid or a pcDNA3 construct that expresses ubc9, the SUMO E2 enzyme. As in the experiments described in Fig. 1 above, the 6-His-APP (V642F) protein was isolated from extracts of the transfected cells using Ni-NTA affinity beads and then analyzed by anti-HA Western blot to detect sumoylated forms of this protein. The results indicate that the amount of sumoylation of this APP protein is higher in the ubc9-overexpressing cells (Fig. 3A), and that this is associated with decreased amounts of A β aggregates in extracts of these cells (Fig. 3C).

Sumoylation of protein domains that are exposed to the lumen of the endoplasmic reticulum (ER) or other compartments of the secretory pathway has not been previously reported. However, the results described above indicating the SUMO modification of APP suggested that sumoylation may be occurring within one or more of these membrane-bound compartments. To test the feasibility of this hypothesis, we performed immunofluorescence analysis of cells using antibodies against the SUMO E2 enzyme, ubc9, as well as the ER protein calnexin. The results indicate that a portion of the ubc9 staining in cells overlaps that of calnexin (Fig. 4A and 4B), suggesting that this sub-population of the SUMO E2 enzyme colocalizes with the ER. The ubc9 staining pattern we observed is consistent with the results of previous studies that examined the subcellular localization of ubc9, which showed that, in addition to a nuclear-localized population, some of the ubc9 staining is found outside the nucleus in a pattern reminiscent of ER localization [9,22,23].

As an independent approach for testing the apparent co-localization of the SUMO E2 enzyme with the ER, Western blot analysis was performed on nuclear vs. ER-enriched vesicle fractions using antibodies against ubc9 as well as antibodies against lamin A and calnexin as positive controls for the nuclear and ER fractions, respectively. The results indicate that a portion of the cellular ubc9 is detected in the ER vesicle fraction, both uncharged ubc9 and ubc9 that is

covalently charged with SUMO, the form of the protein ready to transfer the SUMO group to other proteins (Fig. 4C). The appearance of less ubc9 protein in the ER vesicle fraction vs. the nuclear fraction may be due to the several subsequent differential centrifugation steps required to obtain the ER vesicles, resulting in lower relative yields. The results in Figure 4C support the hypothesis that the SUMO E2 enzyme is associated with the ER.

For ubc9 to be able to sumoylate APP, it would presumably have to be present within the luminal compartment of the secretory pathway. To test this hypothesis we subjected sealed calf brain microsomes enriched in rough endoplasmic reticulum to trypsin digestion in the absence or presence of Triton X-100, with the detergent functioning to unseal the vesicles to expose their contents to the protease. The results reveal that in the absence of Triton X-100 no degradation of ubc9 is observed, but that treatment of the vesicles with this detergent is associated with digestion of the ubc9 protein (Fig. 4D). These results suggest that the SUMO E2 enzyme is found within the lumen of these vesicles. The results also indicate that both uncharged ubc9, as well as a substantial amount of active SUMO-charged ubc9, are found within this compartment.

Discussion

The results in this paper indicate that lysines 587 and 595 of the APP protein are covalently modified by both SUMO-1 and SUMO-2, and that this modification negatively regulates levels of A β aggregates. The mechanism by which sumoylation at lysines 587 and 595 leads to decreased A β aggregate levels is unknown, but the close proximity of the sumoylation sites to the site of β -secretase cleavage suggests the possibility that the presence of the 97 amino acid SUMO protein attached at these sites might sterically block binding of this protease to APP.

One of the lysine residues identified as a sumoylation site by this study, lysine 595, is the same lysine that is mutated to asparagine in the previously characterized Swedish (KM-to-NL) APP mutant [24]. This suggests the possibility that inability to be sumoylated at this lysine could contribute, at least to some extent, to the increased A β production exhibited by the Swedish APP mutant.

Two previous studies obtained data indicating that overexpression of SUMO proteins affects the levels of A β [25,26]. However, these studies did not examine whether APP was sumoylated, and in addition the meaning of these results is not clear as the two studies observed opposite effects of SUMO protein overexpression on A β levels. In any event, it appears these effects of SUMO overexpression on A β are likely mediated by a mechanism different from that of direct APP sumoylation examined in our study as they were observed even when non-conjugatable SUMO proteins were expressed [26].

The data presented in this paper also indicate that the SUMO E2 enzyme ubc9 is present within the lumen of the endoplasmic reticulum, providing an explanation for how lysines 587 and 595 of APP can be sumoylated, and suggesting that other proteins entering the endoplasmic reticulum may also be targets of sumoylation. This result extends the sub-cellular reach of sumoylation to include the regulation of proteins in the secretory pathway. The mechanism by which ubc9 enters this compartment is unclear, as it does not appear to contain any obvious signal sequence. However, ubc9 is identified as a candidate non-classical secretory pathway protein by the prediction program SecretomeP [27]. Thus, one possibility is that ubc9 enters some membrane-bound compartment in the cell which then subsequently merges with the ER.

Finally, the results indicating that elevation of ubc9 expression reduces levels of A β aggregates in cells expressing APP V642F suggest that approaches that up-regulate cellular sumoylation have potential as new therapeutic strategies against Alzheimer's disease. Future studies

exploring this and other questions should reveal new information about APP sumoylation and its potential medical applications.

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Fig. 1. APP is modified by SUMO-1 and SUMO-2 at lysines 587 and 595, which negatively regulates Aβ aggregate levels. (A) Schematic showing locations of matches to the sumoylation site consensus sequence (ΨKXE/D) surrounding lysines 587 and 595 in APP, and the proximity of these two lysine residues to the site of β-secretase cleavage and the Aβ peptide generated from APP. (B) HeLa cells were transfected with 6xHis-APP wildtype or mutant APP (K587R, K595R, or K587, 595R) constructs along with HA-SUMO-1 or HA-SUMO-2 expression plasmids. APP protein was isolated from extracts of the transfected cells by Ni-NTA agarose affinity chromatography, followed by Western blot using anti-HA antibody to detect sumoylated forms of the transfected proteins (top panels). Amount of Aβ aggregates present

in extracts of each transfected cell population was examined by Western blot using anti-A β (1–17) mouse monoclonal antibody (middle panels). As a loading and normalizing control, amount of full-length APP protein in the cell lysate was determined by Western blot using an antibody against the C-terminal region of APP (bottom panels).

Fig. 2.
Endogenous brain APP is sumoylated. Extracts of mouse brain were subjected to immunoprecipitation using an antibody against the C-terminal region of APP, followed by Western blot analysis of the immunoprecipitates using anti-SUMO-1 or anti-SUMO-2 antibodies.

Fig. 3.

Elevated expression of SUMO E2 enzyme decreases A β aggregate levels. (A) HeLa cells were transfected with a 6xHis-APP V642F construct and HA-SUMO-1 plasmid, along with either empty pcDNA3 or a pcDNA3-ubc9 expression construct. APP protein was isolated from extracts of the transfected cells by Ni-NTA affinity chromatography, followed by Western blot using anti-HA antibody to detect sumoylated forms of the transfected APP protein. (B) Up-regulation of ubc9 protein in cells transfected with the pcDNA3-ubc9 plasmid was confirmed by Western blot of cell lysates using anti-ubc9 antibody. (C) Amount of A β aggregates present in extracts of each transfected cell population was examined by Western blot using an anti-A β (1–17) mouse monoclonal antibody. (D) As a loading and normalizing control, the amount

of full-length APP protein in the cell lysate was determined by Western blot using an antibody against the C-terminal region of APP.

Fig. 4.

The SUMO E2 enzyme is present in the ER luminal compartment. (A) HeLa cells were subjected to immunofluorescence microscopy using antibodies against ubc9 (SUMO E2 enzyme) and the ER marker protein calnexin. DNA was visualized by Hoechst 33342 staining. (B) Higher magnification view using the same visualizations in panel A. (C) Nuclei and a membrane fraction enriched in large dense ER vesicles prepared from HeLa cells were subjected to Western blot using antibodies against the SUMO E2 enzyme ubc9, and with antibodies against lamin A and calnexin as positive controls for nuclear and ER compartments, respectively. (D) Calf brain microsomes enriched in rough endoplasmic reticulum were subjected to trypsin digestion in absence or presence of Triton X-100, and then analyzed by

Western blot using anti-ubc9 antibodies. Lane marked “C” represents an aliquot of microsomes used.