Modulation of Aβ generation by small ubiquitin-like modifiers does not require conjugation to target proteins

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The sequential processing of the APP (amyloid precursor protein) by the β- and γ-secretase and generation of the Aβ (amyloid-β) peptide is a primary pathological factor in AD (Alzheimer's disease). Regulation of the processing or turnover of these proteins represents potential targets for the development of AD therapies. Sumoylation is a process by which SUMOs (small ubiquitin-like modifiers) are covalently conjugated to target proteins, resulting in a number of functional consequences. These include regulation of protein–protein interactions, intracellular trafficking and protein stability, which all have the potential to impact on several aspects of the amyloidogenic pathway. The present study examines the effects of overexpression and knockdown of the major SUMO isoforms (SUMO1, 2 and 3) on APP processing and the production of Aβ peptides. SUMO3 overexpression significantly increased Aβ40 and Aβ42 secretion, which was accompanied by an increase in full-length APP and its C-terminal fragments. These effects of SUMO3 were independent of its covalent attachment or chain formation, as mutants lacking the motifs responsible for SUMO chain formation or SUMO conjugation led to similar changes in Aβ. SUMO3 overexpression also up-regulated the expression of the transmembrane protease BACE (β-amyloid-cleaving enzyme), but failed to affect levels of several other unrelated proteins. Suppression of SUMO1 or combined SUMO2 + 3 by RNA interference did not affect APP levels or Aβ production. These findings confirm a specific effect of SUMO3 overexpression on APP processing and the production of Aβ peptides but also suggest that endogenous sumoylation is not essential and likely plays an indirect role in modulating the amyloid processing pathway.

Key words: Alzheimer's disease, amyloid-β peptide, neurodegeneration, sumoylation.

INTRODUCTION

AD (Alzheimer's disease) is a progressive neurodegenerative disorder characterized by the deposition of extracellular amyloid plaques and intraneuronal tangles. The amyloid pathology results from the accumulation of Aβ (amyloid-β) peptides, which are endoproteolytic fragments of the APP (amyloid precursor protein) (for review see [1]). The amyloidogenic pathway is initiated by cleavage of the integral type I transmembrane protein APP by BACE (β-amyloid-cleaving enzyme), at the N-terminal end of the Aβ domain [2]. The membrane-bound C-terminal fragment of APP is then a substrate for γ-secretase cleavage. This high molecular mass secretase complex is composed of presenilin, NCT (nicastrin), APH-1 and PEN-2 (for review see [3]) and is modulated by the recently identified TMP21 [4]. This second proteolytic event releases Aβ40 or the more fibrillogenic Aβ42 peptides. The accumulation of these abnormal filamentous protein deposits is considered to be a primary cause of neuronal death. Mutations in APP or presenilins increase the production of amyloidogenic Aβ peptides and are associated with early onset familial AD (for review see [5]).

Genetic and biochemical studies have demonstrated causative links between amyloid pathology and AD. As a result, therapeutic strategies have been investigated to inhibit the production and deposition of Aβ peptides or enhance their clearance. While a number of advances have been made in this area, a more complete understanding of the signalling pathways that regulate APP processing could lead to novel AD therapies. A rapid and effective means of modulating protein function is by post-translational modification. These include chemical modifications (e.g. phosphate, methyl or acetyl) and also the attachment of other polypeptides such as ubiquitin, which is involved in proteasome-mediated turnover. Sumoylation is a post-translational modification by which SUMO (small ubiquitin-like modifiers) are covalently conjugated to target proteins. Three major SUMO isoforms have been identified in humans, and these display cell type-specific expression levels as well as distinct, although not mutually exclusive, subcellular localizations (for review see [6]). Each isoform is expressed as a precursor protein that undergoes maturation by SUMO C-terminal hydrolases (for review see [7]). The resulting mature protein exposes a C-terminal diglycine motif and enters a multi-step enzymatic pathway that is comparable with ubiquitination (for review see [6]). Mature SUMO is activated by the ATP-dependent formation of a thioester bond between its C-terminal glycine residue and an internal cysteine residue in the heterodimeric SUMO-activating (E1) enzyme SAE1/SAE2 [8]. Activated SUMO is subsequently transferred through a trans-esterification reaction to the unique SUMO-conjugating (E2) enzyme, Ubc9 [9]. The final step is the formation of an isopeptide bond between the C-terminal glycine residue of SUMO and the lysine residue ε-amino group of the target substrate. The specificity and the rate of the conjugation may be modulated

Abbreviations used: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; APPwt, human wild-type APP695; BACE, β-amyloid-cleaving enzyme; CTF, C-terminal fragment; FL-APP, full-length APP; GG-AA, diglycine-to-dialanine mutant; HA, haemagglutinin; HEK-293, human embryonic kidney 293; NCT, nicastrin; SAE, SUMO activating enzyme; SBM, SUMO-binding motif; siRNA, small interfering RNA; SUMO, small ubiquitin-like modifier.

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by SUMO (E3) ligases, a growing number of which have been identified (for review see [10]). Specific proteases can rapidly remove SUMO from substrates, reflecting the highly dynamic and reversible process of sumoylation.

A majority of target lysine residues are defined by a SUMO consensus motif, ΨKXE/D, where Ψ is a hydrophobic amino acid residue, K the target lysine residue, X is any amino acid residue and E/D is either a glutamic or aspartic acid residue [11]. The presence of this motif in the N-terminal domain of SUMO2 and SUMO3 allows the formation of polySUMO chains [12]. However, this recognition motif is not an absolute requirement for sumoylation. This is illustrated by the ability of SUMO1 to form polySUMO1 chains despite the lack of a consensus sequence [13]. The number of known substrates has significantly increased as a result of several recent proteomic investigations [14–18]. The functional consequences of SUMO modification are diverse and include the control of protein–protein interactions, modulation of subcellular localization and regulation of protein stability.

Each of these biochemical events could potentially affect APP metabolism, Aβ production and possibly the progression of AD. This idea is supported by a previous study which indicated that sumoylation may act as a positive and negative regulator of APP processing [19]. However, the mechanism by which this was achieved remains uncertain. Another potential link between SUMO and AD has recently been demonstrated by the site-specific sumoylation of the microtubule-associated tau protein, which is the main component of neurofibrillary tangles [20]. Recent reports have also implicated SUMO in neurodegenerative processes associated with Huntington's disease and related disorders [21–25]. To further explore the effects and mechanisms of SUMO action on APP processing and Aβ production, the present study was undertaken to investigate the overexpression and knockdown of the three principal SUMO isoforms. This revealed that overexpression of SUMO3 significantly up-regulated APP levels and increased Aβ production. These effects may be due to increases in the γ-secretase substrate concentrations or possibly changes in other secretase components. However, the suppression of endogenous SUMOs did not have any significant effect on Aβ secretion.

MATERIAL AND METHODS

Plasmids and siRNAs (small interfering RNAs)

Plasmids encoding N-terminally HA (haemagglutinin)-tagged (peptide YPYDVPDYA) full-length SUMO1, SUMO2 and SUMO3 correspond to the human protein sequences as previously described [12]. The conjugation-deficient SUMO3 double mutant (G92A, G93A) and the polymeric-deficient SUMO3 single mutant (K11R) were generated by site-directed mutagenesis according to the manufacturer's instructions (Stratagene). All SUMO constructs, human wild-type APP695 (termed APPwt) and human wild-type tau 4R2N and NCT cDNAs were cloned into pcDNA3 and α-synuclein was cloned into pcDNA6. A pIRES2 plasmid encoding a C-terminally V5-tagged (peptide GKPIPNPLLGLDST) mouse BACE was provided by Dr N. G. Seidah (Clinical Research Institutes of Montréal, Québec, Canada). All expression plasmids were confirmed by DNA sequencing. SUMO1 siRNA oligonucleotides (target sequence AACUGGGAAUGGAGGAAGAAG) were synthesized by Dharmacon RNA technologies. A single siRNA was used to suppress both SUMO2 and SUMO3 expression (SUMO2 + 3 siRNA) and was purchased from Santa Cruz Biotechnology.

Cell culture, transfection and cycloheximide treatment

HEK-293 (human embryonic kidney 293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum. Cells were transfected in reduced serum medium at approximately 50 % confluency using Lipofectamine™ (Invitrogen), according to the manufacturer's instructions. For each transfection, cells were incubated in the presence of 2.5 μg of plasmid DNA encoding HA-tagged SUMO isoforms in the presence or absence of 2.5 μg of APPwt, BACE or other selected proteins as indicated. Where necessary, the pcDNA3 empty vector was used to bring the total amount of DNA to 5 μg. Following 18 h of transfection, the medium was replaced and cells were incubated for an additional 24 h. At the end of the incubation period, the conditioned medium was collected and assayed for Aβ. Cells were harvested, washed in PBS and processed for Western blot analysis. For cycloheximide studies, APP/SUMO3 transfected cells were incubated in the presence of cycloheximide (30 μg/ml) for 2, 6, 8 and 12 h and APP levels were determined by immunoblotting.

siRNA-based knockdown of SUMO expression was performed in HEK-293 cells stably expressing APPwt. Cells were transiently transfected with either 0.31 μM SUMO1 siRNA or 0.09 μM SUMO2 + 3 siRNA, as described above. Medium was replaced and conditioned for 24 h. In the case of SUMO2 + 3 RNA interference, cells were allowed to grow for an additional 24 h in normal growth medium before changing the medium again for the conditioning period (24 h). Conditioned medium was collected and cells harvested as described above.

Aβ ELISA assay

Human Aβ40 and Aβ42 levels in 24 h conditioned media were specifically determined using a standard sandwich ELISA, as previously described [26]. For each condition, Aβ levels were normalized to the protein content (determined using the Bradford assay). The effect of overexpression or knockdown of each SUMO isoform was compared with control in a paired Student's t test. Results were normalized to controls and values represent the mean $\pm$ S.E.M. of at least three independent experiments.

Western blot analysis

Cells were lysed in RIPA lysis buffer [50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 0.5 % deoxycholate and 1 % Triton X-100] containing complete protease inhibitor cocktail (Roche Applied Science). Lysates were cleared by centrifugation (20000 ***g*** for 15 min at 4 °C) and the protein content was determined using the Bradford assay. Proteins were diluted in sample buffer [62.5 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 50 mM DTT (dithiothreitol) and 0.01 % Bromophenol Blue] and equal amounts of proteins were separated by electrophoresis on precast 4–20 % polyacrylamide gels (Invitrogen) and electrotransferred on to nitrocellulose (Amersham Biosciences). HA epitope-tagged SUMO proteins were detected with an anti-HA antibody (clone 12CA5; Roche Applied Science). Endogenous SUMO proteins were visualized using anti-SUMO1 (GMP1; clone 21C7) and anti-SUMO2 + 3 antibodies (clone NRD.1), with the latter recognizing both SUMO2 and SUMO3 isoforms (Zymed Laboratories). Both the polyclonal [APP/CTF (C-terminal fragment)] and the monoclonal (C.1/6.1) antibodies recognize FL-APP (full-length APP) as well as the CTFs. Horseradish peroxidase-conjugated anti-V5 antibody was purchased from Invitrogen. Anti-α-synuclein (Syn1; clone 42) was obtained from Pharmingen. Anti-tau antibody CP27 was a gift from Dr Peter Davies (Albert Einstein College of Medicine, New York,

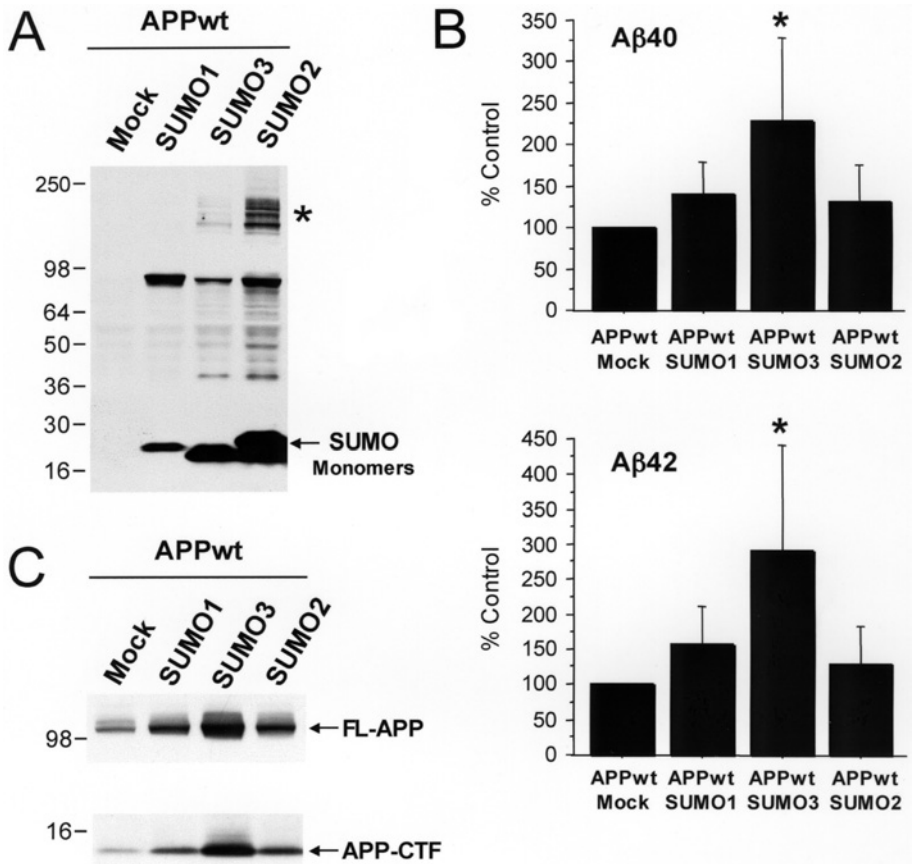

Figure 1 SUMO3 overexpression increases Aβ secretion

HA-tagged full length SUMO isoforms (SUMO1, 2 and 3) were co-transfected with APPwt in HEK-293 cells. (**A**) Cells were harvested, lysed and equal amounts of proteins were analysed by Western blot analysis. SUMO expression and conjugation was visualized using an anti-HA antibody. SUMO monomers (arrow) and the high molecular mass SUMO conjugates (*) were observed. (**B**) Aβ40 and Aβ42 levels in conditioned media were determined by ELISA. Overexpression of SUMO3, but not SUMO1 or SUMO2, significantly increased Aβ secretion [*, significantly different from APP co-transfected with the empty vector (Mock), $P < 0.05$]. (**C**) The levels of both FL-APP and its CTFs were examined by Western blot analysis and overexpression of SUMO3 specifically up-regulated the levels of APP and endoproteolytic CTFs.

NY, U.S.A). NCT was examined using an anti-NCT antibody (clone 35) purchased from BD Transduction Laboratories. The secondary antibodies, horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch. Immunoreactive bands were visualized by using an ECL® detection kit (Amersham Biosciences). All Western blots presented are representative of at least three independent experiments with comparable results.

RESULTS

Effects of elevated SUMO expression on Aβ secretion

The specific effects of SUMO1, SUMO2 and SUMO3 overexpression on APP processing and the production of Aβ peptides were investigated. Native HEK-293 cells were co-transfected with equal amounts of the three individual HA-tagged SUMO isoforms and wild-type human APP695. SUMO expression was visualized by immunoblotting using an anti-HA antibody (Figure 1A). Unconjugated SUMO monomers migrated at ~20 kDa and, for the longest SUMO2 isoform, both the full length and mature processed forms were observed (Figure 1A). This observation may potentially be due to a limiting C-terminal hydrolase activity of a SUMO2-specific protease. Sumoylated substrates typically appeared as high molecular mass species. SUMO2 was expressed at slightly higher levels as compared with the other isoforms. It has been reported that SUMO2 expression levels in HEK-293 cells is lower, which could allow higher expression levels of exogenous transfected proteins [27]. The overall expression and conjugation levels of transfected SUMO1 was somewhat lower as compared with SUMO2 and SUMO3. This corresponded to the pattern of expression for the endogenous proteins in HEK-293 cells, with SUMO1 being the most abundant and there being lower SUMO2 protein levels [16,28]. Lysates from cells transfected with the individual HA-tagged SUMO isoforms were also probed with monoclonal antibodies recognizing SUMO1 and combined SUMO2 + 3. The slight molecular mass differences due to the HA-tag allowed examination of levels of endogenous and transfected proteins. This indicated that the transfected SUMOs were expressed at a comparable level with that of their corresponding endogenous isoform which resulted in an approximately 2-fold increase in total levels (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/404/bj4040309add.htm).

Levels of Aβ40 and Aβ42 in the conditioned media of the double-transient SUMO/APPwt-transfected cells were determined by ELISA (Figure 1B). Overexpression of SUMO1 and SUMO2 had no significant effect on Aβ production. However, SUMO3 overexpression significantly increased both Aβ40 and Aβ42 peptides (~225 % for Aβ40 and ~300 % for Aβ42, $n = 3$, $P < 0.05$) as compared with mock-transfected controls. Although

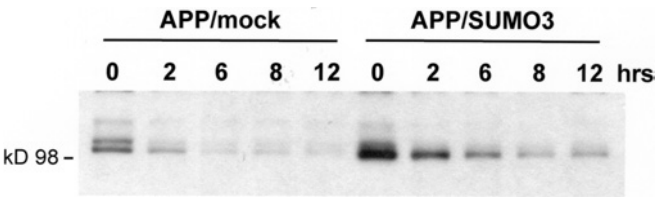

Figure 2 Turnover of APP is affected by SUMO3 overexpression

Cells expressing human APP only and co-transfected with HA-tagged SUMO3 were treated with cycloheximide (30 μg/ml) to eliminate protein synthesis. Lysates were examined by immunoblotting at various time points (0, 2, 6, 8 and 12 h) to determine the levels of FL-APP. Reduced turnover was observed following co-expression of SUMO3.

SUMO3 seemed to have a greater effect on Aβ42 levels, the overall ratio of Aβ42/Aβ40 was not statistically different (mean Aβ42/Aβ40 $\pm$ S.E.M.; 0.073 ± 0.015 for APP/pcDNA3 versus 0.070 ± 0.012 for APP/SUMO3, $P = 0.42$).

The possible effects of SUMO on the levels of the γ-secretase substrates, FL-APP and its CTFs, were also examined. Immunoblotting of lysates from SUMO/APPwt co-transfected cells indicated a substantial increase in APP holoprotein and CTFs upon SUMO3 overexpression (Figure 1C). Co-expression of APPwt and EGFP (enhanced green fluorescent protein), a soluble protein with a molecular mass similar to SUMO, did not significantly affect APP processing, suggesting a specific effect of SUMO3 expression (results not shown). The levels of FL-APP and the CTF were examined by densitometric analysis to determine whether the level of substrate was affected. A significant increase of approximately 2.5-fold in the ratio of CTF/FL-APP was observed for cells transfected with SUMO3 as compared with control (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/404/bj4040309add.htm). This suggested that co-transfection of APP and SUMO3 resulted in enhanced processing of APP to generate the CTF γ-secretase substrate. To examine whether the increase in FL-APP was the result of changes in protein turnover, cells were treated with cycloheximide and FL-APP levels were determined by Western blot analysis. This revealed that APP was degraded at a slower rate when co-transfected with SUMO3 as compared with cells expressing only APP (Figure 2). Detectable levels of the FL-APP were observed even after a 12 h chase but were significantly reduced after only 2 h in the absence of SUMO3 overexpression. This longer half-life was confirmed by densitometric analysis of the Western blot data which indicated that the rate of FL-APP degradation was decreased by approximately 30 % in the presence of SUMO3 (see Supplementary Figure 3 at http://www.BiochemJ.org/bj/404/bj4040309add.htm). Taken together, these data suggest that SUMO3 overexpression and possibly the accompanying up-regulation of sumoylation specifically alters the cellular levels of APP, resulting in an increase in Aβ-generating substrates and secretion of Aβ peptides.

The effects of SUMO3 on expression of other proteins

To ensure that the changes in APP levels were not an artefact of SUMO3 transfection, a number of other related transmembrane and soluble proteins were examined under similar conditions. Cells were co-transfected with V5-tagged BACE and HA-SUMO3. Immunoblotting of cell extracts showed an increase in full length BACE levels similar to that observed for APP (Figure 3A). This suggested that SUMO3 may non-specifically affect transcription of these co-transfected vectors and this would be supported to some extent by the fact that SUMOs have been shown to modulate a number of regulators of gene expression.

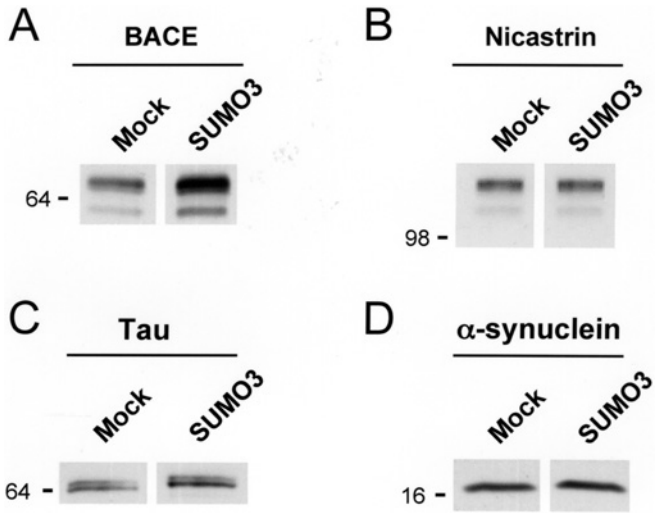

Figure 3 SUMO3 overexpression up-regulates the β-secretase BACE but not NCT, tau or α-synuclein levels

Plasmids encoding (**A**) mouse V5-tagged BACE, (**B**) human NCT, (**C**) human tau or (**D**) human α-synuclein, were co-transfected with HA-tagged SUMO3 in HEK-293 cells. Total cell extracts were analysed by immunoblotting using anti-V5, anti-NCT, anti-tau (CP27) and anti-α-synuclein (Syn1) respectively. An increase in BACE levels was observed upon SUMO3 overexpression while levels of transmembrane NCT, soluble tau and α-synuclein remained unchanged.

This increase in APP substrate (and possibly BACE cleavage) would also provide a direct explanation for the elevated Aβ secretion. However, subsequent investigation of other soluble and membrane proteins indicated that this effect was not a general phenomenon.

A comparable type I transmembrane glycoprotein, NCT, which is part of the γ-secretase complex was also examined. Full-length human NCT was transfected under the same conditions and its level of expression remained unaltered following co-transfection of SUMO3 (Figure 3B). This observation suggested that the observation with BACE did not appear to be a non-specific effect on membrane proteins. The HA-tagged SUMO3 was also transfected with two soluble proteins, tau and α-synuclein, and examined by Western blot analysis. Probing of total cell lysates indicated that there were no detectable changes in expression of either tau or α-synuclein in the presence of similar levels of SUMO3 expression (Figures 3C and 3D). The differences between APP and BACE as compared with the other proteins examined were not the result of transcriptional abnormalities arising from the particular vectors used for transfection [i.e. pcDNA3, pcDNA6 or pIRES2 all under the control of a CMV (cytomegalovirus) promoter; see Materials and methods section]. The specificity of this response is further supported by the recent report of a SUMO3-mediated stimulation of androgen receptor transactivation, which was also independent of the promoters used [29]. Therefore these observations are consistent with a SUMO3-specific effect on the type I transmembrane APP and BACE that was not observed for the soluble proteins. This raises the interesting possibility that SUMO3 may be specifically involved in the trafficking, expression and/or turnover of certain proteins.

SUMO3 mutants and Aβ production

To determine the mechanisms by which SUMO3 increases APP and Aβ, a series of loss-of-function mutants were investigated. Conjugation-deficient mutant were generated by substitution of the C-terminal glycine residues with alanine residues (G92A, G93A). This full length conjugation-defective mutant

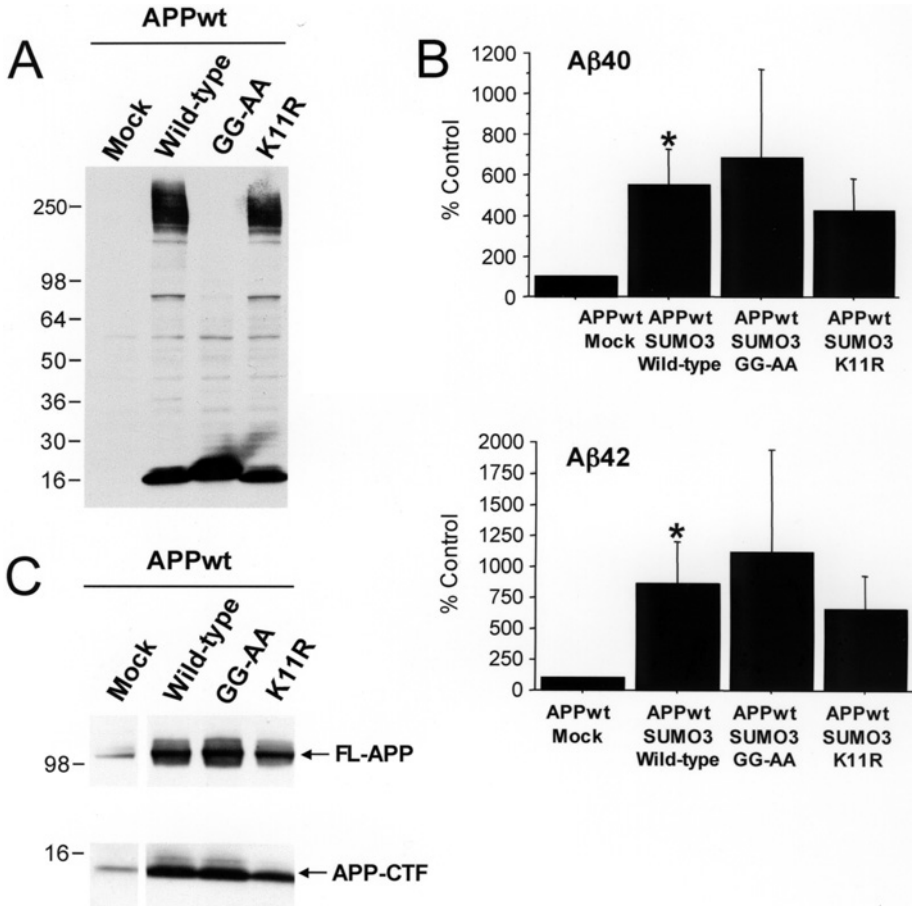

Figure 4 Conjugation- and polymerization-deficient SUMO3 mutants stimulate Aβ production

Plasmids encoding wild-type HA-tagged SUMO3, a C-terminal GG-AA mutant or a K11R mutant were co-expressed along with APPwt. (**A**) SUMO expression and conjugation were analysed by Western blot using an anti-HA antibody. The GG-AA mutant is seen exclusively as a monomer and the absence of high molecular mass species is consistent with its conjugation deficiency. The polymerization-deficient K11R mutant is efficiently conjugated to targets but is unable to form polymeric chains. (**B**) Conditioned media (24 h) were analysed by ELISA as described. Both Aβ40 and Aβ42 levels were increased by wild-type SUMO3 as well as the conjugation- (GG-AA) and polymerization- (K11R) deficient SUMO3 mutants. (**C**) The effect of SUMO3 mutants on APP and its C-terminal fragments was examined by Western blot using an APP/CTF antibody. The levels of both the precursor and the endoproteolytic fragments were up-regulated by the expression of wild-type SUMO3 and the GG-AA and K11R mutants.

cannot undergo maturation and is unable to covalently attach to the target lysine residues. The absence of higher molecular mass conjugates in cells transfected with the SUMO3 GG-AA (diglycine-to-dialanine) mutant and the appearance of predominantly monomeric species was confirmed by immunoblotting (Figure 4A). A slightly higher molecular mass shift of the mutant monomers was also consistent with the retention of the C-terminal residues. SUMOs can also assemble into polymeric chains via conjugation to an internal acceptor lysine residue. Another mutant was generated where the SUMO3 acceptor lysine at residue 11 (Lys^{11}) was substituted with an arginine residue (K11R) to prevent the formation of polySUMO chains. Western blot analysis revealed that the K11R mutant undergoes maturation and was efficiently conjugated to substrates, as evidenced by the appearance of high molecular mass species (Figure 4A). The similarity between the mutant deficient in the formation of polySUMO chains and its wild-type counterpart suggests that polysumoylation is unlikely to account for a large fraction of the total sumoylated pool or is masked by the abundance of other sumoylated substrates. Alternatively, the K11R mutant may be incorporated at the distal end of growing chains using other SUMO isoforms; this notion of mixed SUMO chains has been suggested previously [12].

Cells were transfected with APPwt and the SUMO3 mutants to determine whether these loss-of-function isoforms had any effect on APP levels and/or Aβ production. Medium was conditioned for 24 h and the levels of Aβ40 and Aβ42 were determined by ELISA. When compared with APP/vector-transfected control, overexpression of the conjugation-deficient SUMO3 GG-AA mutant greatly increased the production of both Aβ40 and Aβ42 (Figure 4B). The effect was comparable with cells expressing wild-type SUMO3, although there was greater variability in the levels of secreted Aβ peptides. The increase in Aβ induced by the GG-AA mutant also correlated with an elevated expression of FL-APP and APP-CTFs (Figure 4C), which was comparable with the changes observed for wild-type SUMO3. Similarly, co-expression of APPwt and the SUMO K11R mutant (which is unable to form SUMO polymers) led to an almost identical increase in Aβ and levels of APP and CTFs (Figures 4B and 4C). These results suggest that the overexpression of SUMO3 monomers rather than an overall change in SUMO conjugation or polymerization up-regulates APP/CTF levels and stimulates the

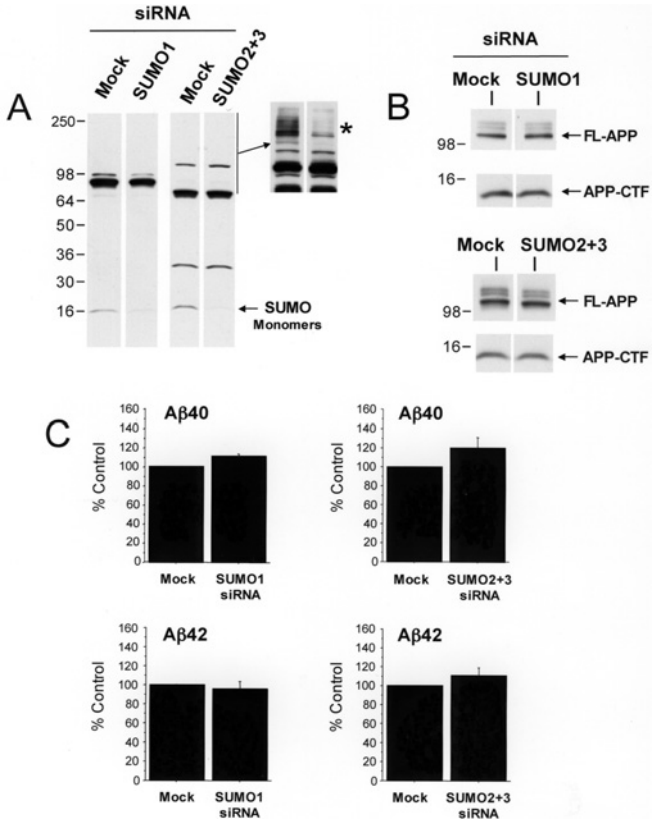

Figure 5 SUMO1 and SUMO2 + 3 knockdowns have no effect on Aβ secretion

SUMO1- and SUMO2 + 3-specific siRNA oligonucleotides were transfected in a HEK-293 cell line stably expressing human APPwt. (**A**) Total cell extracts probed for endogenous SUMO1 or endogenous SUMO2 + 3 using specific antibodies showed a significant decrease in SUMO expression. Longer exposures also revealed that RNA interference suppression of SUMO resulted in decreased conjugation (inset, *). (**B**) Western blot analysis indicated that suppression of SUMOs had no effect on the levels of FL-APP or its CTFs (APP-CTF). (**C**) Conditioned media (24 h) were collected and the levels of Aβ40 and Aβ42 were determined by ELISA. Downregulation of SUMO1 or SUMO2 + 3 expression had no significant effect on the secretion of Aβ40 or Aβ42 peptides.

formation of Aβ peptides. Therefore the findings suggest a non-covalent role for SUMO3 monomers in the observed changes in the amyloid pathway.

Suppression of endogenous SUMO and its effects on Aβ processing

To determine whether endogenous sumoylation plays a role in APP processing and amyloidogenesis, we examined the effect of SUMO knockdown. To avoid interactions between oligonucleotides and plasmids during co-transfection, siRNAs were transfected into a HEK-293 cell line stably expressing human APPwt. A custom-designed SUMO1-specific siRNA was used to negatively regulate SUMO1 expression. Western blot and densitometry analyses showed an approximately 80 % reduction in endogenous SUMO1 monomers as well as a decrease in high molecular mass SUMO1 conjugates (Figure 5A, left panel). Despite a high degree of primary sequence identity (~48 %) between SUMO1 and SUMO2/3, the knockdown was highly specific as no change in the expression and conjugation of other isoforms were detected by Western blot analysis (results not shown). A SUMO2 + 3-specific siRNA was used to concomitantly knockdown SUMO2 and SUMO3 expression. Attempts were made to suppress the individual SUMO2/3 isoforms independently of each other but this was problematical due to their considerable sequence homology (> 95 % identity). However, a robust decrease in the combined SUMO2 + 3 monomers (~94 %) was observed by immunoblotting using an antibody that recognizes both isoforms (Figure 5A, right panel). The conjugation of both isoforms to targets was also significantly reduced, as seen by the decrease in high molecular mass species.

The knockdown of endogenous SUMO1 or combined SUMO2 + 3 had no effect on levels of FL-APP or the CTFs (Figure 5B). Conditioned media were collected and the levels of Aβ peptides were determined by ELISA assay. Under similar conditions, the reduction in SUMO1 or SUMO2 + 3 expression and conjugation did not significantly alter Aβ40 or Aβ42 secretion (Figure 5C). This absence of an effect of SUMO RNA interference suggests that endogenous sumoylation is not an essential regulatory factor but may instead play an indirect role in the processing of APP and production of Aβ peptides.

DISCUSSION

Sumoylation is the process by which SUMO proteins are covalently conjugated to target substrates. This post-translational modification regulates protein interactions, subcellular localization and protein stability. A growing number of reports have demonstrated the co-localization of SUMO with protein inclusions in neurodegenerative diseases such as Huntington's disease and amyotrophic lateral sclerosis [21,30]. Several proteins specifically implicated in these disorders have also recently been shown to be covalently modified by SUMO [20,21,24,30,31].

Using an unbiased screening approach, a previous investigation identified SUMO3 as a modulator of APP processing leading to a decrease in Aβ production [19]. Although this was subsequently revised and noted as SUMO2 [32], the original clone corresponded to a cDNA encoding a full-length 95 residue-SUMO protein. These differences in identification may be due to nomenclature issues, as a sequence length of 95 residues does suggest that the clone was SUMO3 (see sequences as described in [33]). In the present study, we investigated the overexpression and suppression of a collection of SUMO isoforms (SUMO1, 2 and 3) and their effects on APP and Aβ. We observed that overexpression of SUMO3 was the only isoform which significantly altered Aβ levels. In contrast with previous findings [19], we found that transfection of wild-type SUMO3 increased Aβ production. However, the levels of full-length and CTFs of APP were consistently increased [19]. This suggested the possibility that the factors responsible for the endoproteolytic processing and/or turnover of the APP protein substrates may be regulated by sumoylation. This proved to be the case for the β-secretase as shown by co-transfection of BACE and SUMO3, which resulted in an elevation in the secretase levels. This was also found to be SUMO3-specific since similar overexpression of either SUMO1 or SUMO2 had no discernable effect (results not shown). It should be noted that our investigation and others [19–22,24,30] were conducted using transfected cells which have some inherent limitations. This may account for the different observations and the effects on endogenous proteins have yet to be determined. However, the observed changes in APP and its CTFs, as well as amyloid-related proteases, would be consistent with increased substrates and ultimately elevated Aβ peptide secretion.

Elevated APP and BACE levels induced by SUMO3 do not appear to be an artefact of transfection given that, under comparable conditions and similar promoter constructs, there was

no observed alteration in the expression of the soluble and membrane proteins (e.g. tau, α-synuclein and NCT). The specificity of these effects was also not a result of exceptionally high expression of SUMO3. The level of the homologous SUMO2 was higher but this isoform had no discernable effect on Aβ production or APP levels. The SUMO3-specific response illustrates the fact that despite the considerable similarity between the various SUMO isoforms, especially between SUMO2 and SUMO3, they can have widely different functional roles. This may tentatively be explained in part by their distinct but not mutually exclusive subcellular localizations or their interaction with unique substrates [34].

The present study suggests that the response to SUMO3 overexpression involves a non-covalent mechanism of action. This is supported by the fact that similar effects on APP were observed for the conjugation-deficient SUMO3 GG-AA mutant. This mutant was unable to attach to substrates but still increased Aβ levels. The modulation of physiological processes by different SUMO isoforms independently of their conjugation to targets has been reported for dynamin [35], thymine DNA glycosylase [36], androgen receptor [29] and parkin, a protein associated with some cases of Parkinson's disease [37]. Recent reports have identified SBMs (SUMO-binding motifs) [15,34,38,39] which further supports the possibility that interactions with SUMO monomers in the absence of conjugation can have functional consequences. Interestingly, molecular and structural details have recently been presented to explain the specificity of SBMs to specifically bind to distinct SUMO isoforms [34]. Therefore direct and specific interactions of SUMO3 with its target proteins may be responsible for the increased APP and BACE leading to the observed changes in Aβ levels. The reason for the observed reduction in APP and BACE turnover is unclear but this may be mediated by the ubiquitin–proteasome system. Both proteins are known substrates for proteasome-mediated degradation and accumulate in response to proteasome inhibitors [40–42]. Therefore it is possible that SUMO3 may modulate proteasome-mediated degradation leading to a specific reduction in APP and BACE turnover.

Due to the low abundance of SUMO conjugates, most sumoylation studies have relied on overexpression systems. We therefore sought to examine the role of the endogenous SUMOs on APP expression, processing and the production of Aβ peptides. Despite the stability of SUMO proteins [16], the expression and conjugation of SUMO1 and SUMO2 + 3 isoforms were successfully knocked down using an RNA interference approach. However, no effect on APP, its CTFs or Aβ was observed. The possibility that critical SUMO substrates were not affected following SUMO knockdown cannot be excluded. One possible explanation for the lack of effect following suppression as compared with SUMO3 overexpression may be the response of the ubiquitin–proteasome system. For example, suppression by interference may not necessarily translate into proteasome activation. If this is the case, loss of a particular SUMO species may have relatively little effect on ubiquitin conjugation, resulting in normal proteasome function and no change in amyloid processing.

A growing body of evidence suggests a role for SUMO proteins in neurodegenerative diseases. The present study has demonstrated a specific conjugation-independent SUMO3-mediated increase in APP, BACE and Aβ levels. These responses may be the result of changes in protein turnover by the proteasome, or related protein degradation pathways, or possibly by alterations in protein trafficking. If this model holds true, it may provide additional insight into the cellular pathology of AD and related disorders.

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