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Presenilins are novel substrates for TRAF6-mediated ubiquitination

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ABSTRACT

Mutations in presenilins (PS1 and PS2) have been linked to the pathogenesis of early onset familial Alzheimer's disease. Presenilins function as the catalytic component of the γ-secretase protease complexes responsible for the cleavage of the amyloid precursor protein (APP), subsequent generation of amyloid-β and associated amyloid plaques in Alzheimer's disease. Biochemical and genetic studies have revealed that through interactions with several proteins, the presenilins are functionally involved in a range of cellular processes, including the regulation of intracellular calcium homeostasis. Our group has previously reported an association between presenilins and members of the tumour necrosis factor receptor-associated factor (TRAF) family of proteins. In this study we further investigated the association between TRAF6, an E3 ubiquitin ligase, and the presenilins. Here we show that the presenilin full-length holoproteins are novel substrates of TRAF6-mediated Lysine-63-linked ubiquitination. Interestingly, co-expression of catalytically active TRAF6 with the presenilins leads to decreased turnover of PS1 full-length holoprotein accompanying elevated presenilin protein levels. Similarly, while overexpression of TRAF6 increases presenilin holoprotein levels and ubiquitination in HEK293 cells, expression of catalytically deficient TRAF6 or TRAF6-deficiency leads to a reduction in presenilin protein levels and reduced PS1 ubiquitination. We also demonstrate that TRAF6 induces PS1 gene transcription in a JNK-dependent manner. Notably, we reveal that TRAF6-mediated ubiquitination of presenilin does not affect γ-secretase enzyme activity, but may regulate presenilin function in calcium signalling. Taken together, we propose that presenilins are novel substrates for TRAF6-mediated K63-linked ubiquitination and that ubiquitination of presenilins by TRAF6 increases presenilin holoprotein levels and in conditions in which TRAF6 ubiquitination of presenilins is reduced results in reduction of calcium release from the endoplasmic reticulum.

1. Introduction

Alzheimer's disease (AD) is characterised by the formation of intracellular neurofibrillary tangles and extracellular accumulation of amyloid-β (Aβ) peptides in neuritic plaques in specific brain regions and cerebral vasculature [1–3]. The majority of early onset familial AD (FAD) cases has been linked to mutations in presenilins (PS1 and PS2), which cause a shift in the ratio of Aβ peptides, $A\beta_{42}$:$A\beta_{40}$, resulting in accumulation of the more amyloidogenic $A\beta_{42}$ peptides [4]. Following endoproteolytic cleavage and formation of N- and C-terminal fragments (NTF and CTF) [5], presenilin functions as the catalytic subunit of the γ-secretase protease complexes, comprising presenilins, Nicastrin, anterior pharynx defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2) [6]. In excess of 100 different type-I integral membrane proteins have been reported as proteolytic substrates of γ-secretase, including the widely studied amyloid-β precursor protein (APP) and Notch receptor, suggestive of a regulatory role for presenilins in several signalling events. Knockout of the two related genes (*PSEN1* and *PSEN2*) encoding PS1 and PS2 results in complete ablation of γ-secretase activity and inhibition of Aβ peptide generation [7]. Notably, knockout of *PSEN1* alone is embryonic lethal, while animals deficient in *PSEN2* are viable and fertile, clearly indicating functional distinctions between PS1 and PS2 [8,9]. A growing body of evidence also indicates that presenilins function in several intracellular signalling pathways, independent of γ-secretase activity, most notably intracellular calcium signalling mechanisms, particularly endoplasmic reticulum (ER) calcium homeostasis. The ER functions as an important store for signalling calcium where the concentration of calcium in the ER is nearly 1000-fold higher than that of the cytosol. Uncleaved full-length presenilin holoproteins are approximately 50 kD in size and are primarily found on the ER membrane. It was recently demonstrated that presenilins can form low-conductance

Abbreviations: AD, Alzheimer's disease; AICD, APP intracellular domain; APH-1, anterior pharynx-defective-1; APP, Amyloid precursor protein; Aβ, Amyloid β; C99, 99-amino acid APP C-terminal fragment; ER, Endoplasmic reticulum; FAD, Familial Alzheimer's disease; HEK293T, Human Embryonic Kidney 293 T; IP, Immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; MEF, murine embryonic fibroblast; NEMO, NF-κB essential modifier; NF-κB, nuclear factor-kappa B; PEN-2, presenilin enhancer-2; PS1, presenilin1; PS2, presenilin2; RING, Really interesting new gene; TRAFs, Tumour necrosis factor receptor-associated factors.

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divalent-cation-permeable ion channels and that presenilins account for ~80% of passive Ca^{2+} leak from the ER [10], and many familial Alzheimer's disease presenilin mutations impair this function [11–13].

In addition to endoproteolysis, presenilins are also regulated by diverse posttranslational modifications including phosphorylation [14–16] and ubiquitination [17,18]. These modifications are not only essential for the stability and activation of presenilins, but are also important for the protease assembly and activity of γ-secretase complexes. For instance, SEL-10, a member of the SCF (Skp1-Cdc53/CUL1-F-box protein) E2-E3 ubiquitin ligase family was shown to interact with and enhance PS1 ubiquitination, and alter the cellular levels of PS1 holoprotein and its NTF/CTF heterodimers [18]. Previously, we have also reported the association between PS1 and another E3 ligase, tumour necrosis factor receptor-associated factor 6 (TRAF6) [19].

The TRAF family of adapter proteins has been shown to play an important role in several growth factor and cytokine signalling pathway, such as mitogen-activated protein kinases (MAPK), nuclear factor-kappa B (NF-κB), and PI3K/Akt, in response to microbial, growth factor and cytokine stimuli [20,21]. Seven members of the TRAF family (TRAF1-TRAF7) have been identified and share a common structural domain, the C-terminal homology region, which is able to bind to the cytoplasmic domain of receptors and other TRAF proteins. All TRAF proteins, except TRAF1, also contain a N-terminal RING domain common to E3 ubiquitin ligases, followed by several zinc fingers. The RING domain and zinc finger domains are essential for the activation of downstream signalling cascades [21]. Within the TRAF family, TRAF2, TRAF5 and TRAF6 are reported to possess E3 ligase activity [22]. TRAF6 has been shown to undergo lysine-63 (K63)-linked auto-ubiquitination [23], and to facilitate a diversity of signalling pathways by catalysing K63 linked ubiquitination of specific substrates [24]. Unlike K48-linked polyubiquitination, which targets proteins for proteasomal degradation, K63-linked polyubiquitination does not degrade targeted proteins, but activates signalling pathways. Notably, TRAF6 also promotes K6, K27 and K29 linked ubiquitination of amino terminal fragments of huntingtin protein associated with the pathogenesis of Huntington's disease [25].

In this study, we report for the first time that presenilin holoproteins are substrates of TRAF6-mediated K63-linked polyubiquitination and that ubiquitination of presenilins by TRAF6 alters presenilin holoprotein levels and this post-translational modification regulates presenilin holoprotein functions in calcium signalling, independent of γ-secretase activity.

2. Experimental procedures

2.1. Expression vector construction

The PS1, PS2, FLAG-TRAF6, FLAG-TRAF6 DN, FLAG-TRAF6 K124R and FLAG-TRAF6 C70A expression constructs were described previously [19,26]. HA-ubiquitin and HA-ubiquitin K63R were generous gifts from Dr. R. Carmody (University of Glasgow). PS1 NTF, PS1 CTF and APP CT100 were gifts from Scios Incorporation.

2.2. Antibodies and reagents

All salts, reagents and anti-Flag agarose beads were purchased from Sigma-Aldrich unless otherwise stated. Protein G-sepharose beads were procured from Invitrogen. Complete protease inhibitor tablets were purchased from Roche. All antibodies were obtained from commercial sources: Rat anti-human PS1-NTF and anti-human PS1-CTF were purchased from Chemicon; anti-HA were purchased from Covance; anti-β-actin, anti-β-Tubulin, anti-APP CTF and anti-FLAG were purchased from Sigma-Aldrich; anti-p-JNK, anti-JNK1, anti-TRAF6, anti-Aph-1 and anti-Pen-2 were purchased from Santa-Cruz Biotechnology; polyclonal rabbit anti-human PS2 was obtained from Cell Signalling Technology; and anti-Nicastrin was purchased from BD biosciences. Infrared secondary antibodies IRDye® 800 Goat Anti-Rabbit IgG and IRDye® 800CW Goat Anti-Mouse IgG were purchased from Licor Biosciences.

2.3. Cell culture

Human Embryonic Kidney 293T (HEK293T) cells were originally purchased from ATCC. Presenilin deficient Murine embryonic fibroblast (MEF) cells were gifts from Dr. Bart De Strooper and TRAF6 deficient MEFs were gifts from Dr. Tak Mak. HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM-21) containing 10% foetal bovine serum, 1% L-glutamine, and antibiotics (50units/ml penicillin and 50 μg/ml streptomycin). Murine embryonic fibroblast (MEF) was grown in DMEM-21 complemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and penicillin/streptomycin. Cells were maintained in a humidified 37 °C incubator with 5% CO_2. Transfection was carried out on adherent human HEK293T cell cultures using the calcium phosphate precipitation method. Transfection of MEF cells was carried out by using GeneJuice Transfection Reagent (#70967) from Merck Biosciences following manufacturer's directions.

2.4. Co-immunoprecipitation and Western blot analysis

24–48 h after transfection, cell cultures were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM sodium orthovanadate and protease inhibitor mixture (Complete™, Roche Molecular Biochemicals)). Cells were lysed on ice for 30 min, spun at 13,000 rpm at 4 °C for 10 min and supernatants were collected. Lysates were normalised using the bicinchonic acid (BCA) method by Pierce. Equivalent concentrations of lysates were pre-cleared for 1 h at 4 °C with 25 μl Protein-G sepharose beads. Pre-cleared lysates were immunoprecipitated overnight at 4 °C with 2–5 μg of the indicated antibody followed by incubation with 25 μl Protein-G sepharose beads for 3 h. Immunoprecipitates were then washed two times in 500 mM NaCl lysis buffer followed by one wash in 150 mM NaCl lysis buffer. Washed Protein G beads were boiled in SDS loading buffer for 5 min on a heating block. Samples were resolved on 10% or 12% SDS-PAGE gels, transferred to nitrocellulose membrane (Schleicher and Schuell Bioscience). Following transfer, membranes were blocked for 1 h at room temperature in 5% Marvel milk/TBS-Tween 0.1%. Blots were incubated with indicated primary and secondary antibodies sequentially and proteins were detected with the Licor Odyssey Infrared Imaging System. For detection of exogenous or endogenous expression of proteins, cells were lysed as described above and then equivalent amount of proteins was subjected to SDS-PAGE gel. Blots were incubated with indicated antibodies and were visualised as described above.

2.5. Ubiquitination assays

Transient transfection was performed to HEK293T cells by calcium phosphate precipitation with indicated constructs. Thirty-six hours after transfection, cell cultures were washed twice in ice-cold PBS and detached from plates by gentle scraping in 1 ml PBS-EDTA (0.5 mM). Cells were harvested by spinning at 1000 rpm for 5 min at 4 °C. Pellets were resuspended in 250 μl 1% (w/v) Sodium Dodecyl Sulphate (SDS) and protease inhibitors and boiled for 5 min on a heating block. Following cooling on ice, an equal volume of ice-cold covalent buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate and protease inhibitors) was added, lysate was mixed and spun at top speed at 4 °C for 20 min. The pellet was removed and lysates were normalised using the BCA method. Samples were then subjected to immunoprecipitation with indicated antibodies as described previously. Protein-G sepharose beads were washed three times in covalent buffer and boiled in SDS sample buffer