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A ubiquitin-binding CUE domain in presenilin-1 enables interaction with K63-linked polyubiquitin chains

Stephen P. Duggan [1], Run Yan [1], Justin V. McCarthy *

Signal Transduction Laboratory, School of Biochemistry and Cell Biology, ABCRF, Western Gateway Building, Western Road, University College Cork, Cork, Ireland

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ABSTRACT

The presenilins (PS1 and PS2) are the catalytic component of the γ-secretase intramembrane protease complex, involved in the regulated intramembrane proteolysis of numerous type I transmembrane proteins, including amyloid precursor protein (APP) and Notch. Herein, we describe the identification and characterization of a CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation) ubiquitin-binding domain (UBD) in PS1, and demonstrate that the CUE domain of PS1 mediates non-covalent binding to Lysine 63-linked polyubiquitin chains. Our results highlight a γ-secretase-independent function for non-covalent ubiquitin signaling in the regulation of PS1, and add new insights into the structure and function of the presenilin proteins.

1. Introduction

The reversible covalent attachment of the 8kDa protein ubiquitin (Ub) to cellular proteins, predominantly to primary amines (ε-amino groups of Lys and to the N termini of proteins) targets proteins for proteasomal degradation and also facilitates several non-proteasomal functions in the assembly, amplification and transmission of intracellular signals [1–4]. Protein ubiquitination is the culmination of a multistep process involving three classes of enzymes, known as ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2s), and substrate-specific ubiquitin protein ligases (E3) [4]. Proteins may be ubiquitinated on a single lysine, resulting in monoubiquitination or on several lysine residues resulting in multiubiquitination. In addition, some E2/E3 combinations can then use lysines on the substrate-conjugated ubiquitin to act as acceptors during sequential rounds of ubiquitination, resulting in substrate polyubiquitination [5]. Ub contains seven lysines, which can be utilized during polyubiquitin chain formation and depending on which of the seven lysines act as the acceptor, the ubiquitin chains will have different types of linkages with diverse conformations and create a diversity of molecularly distinct signals in the cell [4,6]. The range of substrate-ubiquitin structures is important for the targeting of ubiquitinated substrates to different fates. For example, K11- and K48-linked polyubiquitin chains generally target proteins for proteasomal degradation [7], while K63-linked chains can regulate kinase activation, DNA damage tolerance, signal transduction, and endocytosis [8,9].

Modulation of protein–protein interactions is an important mechanism involved in the assembly, amplification and transmission of intracellular signals. The recognition of ubiquitin and polyubiquitin chains by ubiquitin-binding domains (UBDs) is critical for determining the outcome of ubiquitination and subsequent ubiquitin-mediated signaling pathways [2]. To date, approximately 20 different UBDs have been identified, including ubiquitin interacting motifs (UIMs), ubiquitin associated domains (UBAs) and the related coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domains [10–12]. The UBA and CUE domains are similar in size, approximately 40 residues, and show a common structural homology consisting of a three helical bundle [10]. The CUE domain was initially characterized as an ubiquitin-binding motif and named for the yeast Cue1p protein, which is essential for the targeting of ubiquitinated protein to degradation pathways [13–15]. Functional studies subsequently revealed that CUE domains promote the ubiquitination of the proteins that

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* Corresponding author at: School of Biochemistry & Cell Biology, ABCRF, 3.41 Western Gateway Building, Western Road, University College Cork, Cork, Ireland. Fax: +353 21 490 1382.

E-mail address: jv.mccarthy@ucc.ie (J.V. McCarthy).

[1] These authors contributed equally to this work.

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contain it [15]. Since then, CUE domains have also been reported to contribute to the stability and specificity of the CUE–ubiquitin complex, and moreover that CUE domain containing proteins play an important role in stabilizing its binding partner [16,17]. The CUE domain of other proteins has been shown to mediate interactions between ubiquitin and CUE domain containing proteins, which facilitates their monoubiquitination [18–20].

The presenilins, PS1 and PS2, are highly conserved transmembrane proteins that are synthesised as 50kDa holoproteins that undergo endoproteolysis to generate heterodimeric presenilin N-terminal and C-terminal fragments (NTF/CTF) that form the catalytic subunit of the γ-secretase protease complex. Initially characterized as a protease responsible for the cleavage of amyloid precursor protein (APP) and the generation of amyloid-β peptides, the γ-secretase protease is now known to be responsible for the cleavage of numerous type I transmembrane proteins associated with several developmental and cellular processes, including the Notch receptor, ErbB4, Cadherins, Insulin-like growth factor receptor (IGFR1) [21] and the interleukin-1 receptors, IL-1RI [22,23] and IL-1RII [24]. In addition to the well-researched role of presenilins as the catalytic core of the γ-secretase protease, the involvement of presenilin holoproteins in the regulation of intracellular calcium homeostasis has become a focus of presenilin and Alzheimer's disease research [25–28].

Critical to the complexity of presenilin-associated activities, PS1 and PS2 are differentially controlled by post-translational modifications including endoproteolysis, caspase cleavage, phosphorylation and ubiquitination [29]. These modifications determine subcellular localisation [30], selectivity of binding-partners, rate of protein turnover [31] and γ-secretase protease activity [32,33]. PS1 is ubiquitinated by SEL-10 and tumor necrosis factor receptor associated factor 6 (TRAF6), which regulate γ-secretase activity and calcium homeostasis [34], respectively. Here we report the identification and characterization of a ubiquitin binding CUE domain in PS1, which facilitates the binding of PS1 to K63-linked polyubiquitin chains.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T and presenilin-deficient murine embryonic fibroblasts (MEFs) cells were maintained in Dulbecco's modified Eagle's medium (DMEM-21) supplemented 10% fetal bovine serum at 37 °C. Transfection of HEK293T and MEFs was performed using the calcium phosphate precipitation method and TurboFect™ (BioRad Laboratories), respectively.

2.2. Expression vectors construction

The pcDNA3.1-PS1 expression construct was described previously [35,36]. PS1ΔCUE, PS1F283A/P284A and PS1V309A/S310A, were generated by site-directed mutagenesis using QuickChange Site Directed Mutagenesis Kit (Stratagene) and the following primers: PS1ΔCUE: GATTTAGTGGCTTATAATGCAGAA AGCACAGAA (sense) and TTCTGCATTATAAGCCACTAAATCATATAC TGA (antisense); PS1: CGGGGTACCGCCATGACAGAGTTACCTGCA CCGTTGTCC (sense) and CCGGAATTCCTAGATATAAAATTGATGGA ATGCTAATTG (antisense); PS1F283A/P284A: GAGAAATGAAACGCT TGCTGCAGCTCTCATTTACTCC (sense) and GGAGTAAATGAGAG CTGCAGCAAGCGTTTCATTTCTC (antisense); PS1V309A/S310A: GGAAGCTCAAAGGAGAGCAGCCAAAAATTCCAAG (sense) and CTTGGAATTTTTGGCTGCTCTCCTTTGAGCTTCC (antisense). Position of mutated sites are underlined. GST-fusion proteins, GST-PS1 loop domain (residues 265–380), GST-PS1F283A/P284A and GST-PS1V309A/S310A loop domain mutants were amplified by PCR (KOD polymerase, Novagen) using CGGGGATCCGCCGTTTT GTGTCCGAAAGGT (sense) and CCGGAATTCCTATTTTACTCCCCTTT CCTC (antisense) and subcloned into pGEX-6P-1 GST expression vector (GE Healthcare). HA-ubiquitin, HA-ubiquitin $K48_{only}$ and HA-ubiquitin $K63_{only}$ were a generous gift from Dr. R. Carmody (University of Glasgow). HA-P62 was a gift from Prof. Jorge Moscat & Dr. Marie Wooten (Sanford Burnham Medical research Institute, CA), and the HA-P62 F406V mutant was generated by site-directed mutagenesis using previously published primer pairs [37]. The NEXT (Notch extracellular truncation) expression plasmid was a gift from Raphael Kopan (Washington University) [38]. APP-CT100 was a gift from Scios Inc.

2.3. Western blot analysis

Total or immunoprecipitated protein extracts were obtained from cells 24–48 h after transfection with specific plasmids. Cells were lysed (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)) and protein concentrations in the extracts were measured with a bicinchoninic acid assay (Pierce). Equal amounts of extracts were resolved on 6%, 10%, 12% or 15% SDS–PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with anti-PS1 NTF [39], anti-PS1-NTF (Chemicon), anti-HA (Covance), anti-APP (Sigma Aldrich), anti-Ubiquitin (P4D1, Santa Cruz Biotechnology), anti-β-actin (Sigma Aldrich) and anti-cleaved Notch 1 (Val 1744) (Cell Signaling Technology) antibodies, followed by incubation with secondary horseradish peroxidase-labeled anti-mouse, anti-rabbit (Dako), or infrared secondary antibodies IRDye® 800 Goat Anti-Rabbit IgG or IRDye® 800CW Goat Anti-Mouse IgG (Licor Biosciences).

2.4. In vitro ubiquitin binding assay

For the whole-cell ubiquitin binding studies, cells expressing the indicated proteins were lysed under stringent denaturing conditions and immunopurified with anti-P62, or PS1 antibodies and protein G-sepharose beads (Invitrogen) and subsequently washed 3 times with covalent buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton, 0.1% SDS, 0.5% sodium deoxycholate, 15 mM NEM and protease inhibitors) and once with binding buffer (20 mM Tris–HCl (pH 7.6), 50 mM NaCl, 0.1% NP40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Samples were incubated overnight at 4 °C in binding buffer with 5 μg of polyubiquitin (K48- or K63-linked) (Boston Biochem), washed with binding buffer and loaded onto a 10% SDS–PAGE, recombinant polyubiquitin (K48- or K63-linked) (1 μg) was used as a positive control, followed by Western blotting analysis using an antibody directed against ubiquitin (P4D1, Santa Cruz Biotechnology).

For the in vitro ubiquitin binding assays, recombinant (20 μg) GST-PS1 loop domain, GST-PS1F283A/P284A or GST-PS1V309A/S310A loop domain mutants were immobilized onto glutathione agarose beads (Sigma Aldrich) and incubated with 800 μg total protein extracted from HEK293T cells, either non-transfected or overexpressing HA-Ub-$K48_{only}$ or HA-Ub-$K63_{only}$ lysed in binding buffer. Samples were incubated overnight at 4 °C, washed in binding buffer and loaded onto SDS–PAGE followed by Western blotting analysis with an antibody directed against ubiquitin (P4D1, Santa Cruz Biotechnology).

2.5. Expression and purification of GST-tagged recombinant proteins

The pGEX-6P-1 GST-PS1 loop domain (residues 265-380), pGEX-6P-1 GST-PS1F283A/P284A and pGEX-6P-1 GST-PS1V309A/