FEBS Open Bio 5 (2015) 763–773

FEBS openbio

journal homepage: www.elsevier.com/locate/febsopenbio

Analysis by a highly sensitive split luciferase assay of the regions involved in APP dimerization and its impact on processing

Marie Decock [a], Laetitia El Haylani [a], Serena Stanga [a], Ilse Dewachter [a], Jean-Noël Octave [a], Steven O. Smith [b], Stefan N. Constantinescu [c], Pascal Kienlen-Campard [a,*]

[a] Institute of Neuroscience, Université catholique de Louvain, Brussels 1200, Belgium
[b] Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794-5215, USA
[c] de Duve Institute and Ludwig Institute for Cancer Research, Université catholique de Louvain, Brussels 1200, Belgium

ARTICLE INFO

Article history:
Received 4 June 2015
Revised 7 August 2015
Accepted 1 September 2015

Keywords:
Alzheimer disease
APP
Dimerization
GXXXG motifs
Amyloid beta peptide
Split luciferase

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease that causes progressive loss of cognitive functions, leading to dementia. Two types of lesions are found in AD brains: neurofibrillary tangles and senile plaques. The latter are composed mainly of the β-amyloid peptide (Aβ) generated by amyloidogenic processing of the amyloid precursor protein (APP). Several studies have suggested that dimerization of APP is closely linked to Aβ production. Nevertheless, the mechanisms controlling APP dimerization and their role in APP function are not known. Here we used a new luciferase complementation assay to analyze APP dimerization and unravel the involvement of its three major domains: the ectodomain, the transmembrane domain and the intracellular domain. Our results indicate that within cells full-length APP dimerizes more than its α and β C-terminal fragments, confirming the pivotal role of the ectodomain in this process. Dimerization of the APP transmembrane (TM) domain has been reported to regulate processing at the γ-cleavage site. We show that both non-familial and familial AD mutations in the TM GXXXG motifs strongly modulate Aβ production, but do not consistently change dimerization of the C-terminal fragments. Finally, we found for the first time that removal of intracellular domain strongly increases APP dimerization. Increased APP dimerization is linked to increased non-amyloidogenic processing.

1. Introduction

The amyloid precursor protein (APP) is a ubiquitously expressed type 1 transmembrane protein [1,2]. APP undergoes proteolysis via two distinct pathways known as the amyloidogenic and the non-amyloidogenic pathways. APP processing is initiated by the shedding of the large ectodomain by either an α-secretase (non-amyloidogenic pathway) or the β-secretase BACE1 (amyloidogenic pathway). APP β-cleavage generates a membrane-anchored β C-terminal fragment (βCTF or C99), which is further cleaved by the γ-secretase complex to generate the Aβ peptides. The 40 and 42 amino acids Aβ isoforms (Aβ40 and Aβ42, respectively) are the major constituents of the senile plaques, a typical lesion found in the brain of patients with Alzheimer's disease (AD) [4]. Mutations responsible for inherited or familial AD cases (FAD) are located in the APP or presenilin genes (PS1 and PS2). The presenilin proteins are the catalytic subunits of the γ-secretase. AD mutations typically result in an increased Aβ42/Aβ40 ratio [2]. Imbalanced production of Aβ, along with its aggregation and accumulation in the brain, may therefore play a critical role in the onset and progression of AD [5].

Although Aβ involvement in the pathology has been extensively studied over the past decades, our knowledge of the physiological function of APP and the cellular mechanism regulating its processing remain remarkably incomplete. APP belongs with its two paralogs APLP1 and APLP2 to the APP-like protein family. Unique features in each member of the family could account for its specialized and specific function [6]. Ten APP isoforms generated by

Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; AICD, APP intracellular domain; sAPPα, soluble APPα; sAPPβ, soluble APPβ; CHO, chinese hamster ovary; CTF, C-terminal fragment; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; DTT, dithiothreitol; ECL, enzymatic chemi-luminescence; ECLIA, electro-chemiluminescence immuno-assay; FBS, fetal bovine serum; FRET, fluorescence resonance energy transfer; KPI, Kunitz-type protease inhibitor; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffered saline; PS1/PS2, presenilin1/presenilin2; RLU, relative light unit; SP, signal peptide; TM, transmembrane; YFP, yellow fluorescent protein

* Corresponding author at: Université catholique de Louvain, Institute of Neuroscience IoNS/CEMO, Avenue Mounier 53 box B1.53.02, B-1200 Brussels, Belgium. Tel.: +32 2 764 93 35; fax: +32 2 764 54 60.

E-mail address: pascal.kienlen-campard@uclouvain.be (P. Kienlen-Campard).

http://dx.doi.org/10.1016/j.fob.2015.09.002
2211-5463/

alternative splicing of the APP transcript have been identified [3]. The major ones (APP695, APP751 and APP770) differ in their extracellular domain by a Kunitz-type protease inhibitor (KPI) domain present in non-neuronal isoforms (APP751 and APP770), but absent in the neuronal one (APP695).

APP has been proposed to mediate dendritic spine arrangement, neural cell migration and synapse formation, including neuromuscular junctions formation [7] that could underlie the neuromuscular phenotype observed in APP knock-out mice [8]. The possible physiological role of APP can be related to its structural properties. APP resembles a transmembrane receptor with an extracellular region displaying features of a cell surface receptor or an adhesion molecule [9,10]. These different functional regions include copper binding and growth factor-like domains and are required for homo- and heterophilic interactions [11,12]. For instance, APP has been shown to interact with Notch [11,13], another major γ-secretase substrate, and even with the Aβ peptide generated by its processing [14]. APP dimerization is therefore likely to play a pivotal role in its processing and function. Recent studies have indicated that APP dimerization involves both the E1 and KPI regions of the ectodomain [11,15–17] and GXXXG motifs of the transmembrane (TM) domain [18,19]. GXXXG motifs are structural determinants favoring close apposition of TM helices and formation of TM dimers [20,21,38]. The TM region of APP contains three consecutive GXXXG motifs. The FAD A21G mutation (Aβ numbering) known as the Flemish mutation [24] extends the GXXXG interface by adding a fourth GXXXG motif and triggers Aβ production. This strongly suggests that TM interactions are involved in pathophysiological processing of APP [19,23,25,26,33]. It is of particular interest to clearly establish the relation between APP dimerization and its processing, especially its cleavage at the γ site. Studies on APP dimerization have largely been carried out using biochemical approaches (crosslinking, co-immunoprecipitation) [27] focused on TM domain interactions in reconstituted micelles or membrane bilayers [22] or have used purified peptides for structural approaches [27–30]. Very few studies have addressed APP dimerization in living cells. Although split fluorescent proteins assays [13,31,32] have revealed a positive role of the KPI domain in APP dimerization, the role of TM dimerization has appeared much more controversial. It has even been suggested that the TM domain plays only a marginal role in full-length APP dimerization [9,27].

Indeed, the extent of APP and CTFs dimerization in living cells is poorly known. There is no information about the respective contribution of its 3 major domains, and especially of its intracellular domain in this process. The link between APP dimerization and processing is controversial [27,36]. Here we use a new dynamic and highly sensitive split protein assay, the split luciferase assay [37] to define the role and the contribution of the different APP regions to dimerization and clarify the correlation between its dimerization and processing. Our major findings are that full-length APP forms more dimers than APP β and αCTFs. Mutations in the GXXXG motifs, including FAD mutants (Flemish), do not consistently alter dimerization. Strikingly, deletion of intracellular domain strongly favors dimerization. Finally, we found that the extent of dimerization is not correlated to Aβ production, but that increased dimerization observed with APP lacking its intracellular region is linked to increased non-amyloidogenic processing.

2. Material and methods

2.1. Chemicals and reagents

Restriction enzymes, Taq DNA polymerase, all culture media, penicillin-streptomycin solution and Lipofectamine® transfection reagent, Nu-Page® Novex® 4–12% Bis-Tris gels and buffers were from Life Technology Corporation (Carlsbad, CA). Fetal bovine serum (FBS) for culture media was purchased from Thermo Scientific (Rockford, IL). Transfection reagent Trans-IT2020 was from Mirus Bio Corporation (Madison, WI). Analytical grade solvents, salts and poly-L-lysine were from Sigma-Aldrich (St Louis, MO). *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-butyl ester (DAPT) was from Calbiochem (Camarillo, CA). Protease inhibitor cocktail was purchased from Roche (Basel, Switzerland). BCA protein assay kit was from Pierce (Rockford, IL, USA). Nitrocellulose membranes were obtained from GE Healthcare (Fairfield, CT). ECL reagents were obtained from Perkin Elmer Inc. (Waltham, MA). *Gaussia* luciferase substrate Coelenterazine native was purchased from Prolume® Ltd. (Pinetop, AZ). The luciferase cell lysis buffer was from New England Biolabs (Ipswich, MA). The following primary antibodies were used: anti-amyloid β antibody, clone W0-2 (EMD Millipore, Billerica, MA), anti-amyloid precursor protein, C-terminal antibody (Sigma-Aldrich, St Louis, MO), anti-GLuc antibody (New England Biolabs, Ipswich, MA). Fluorescent nucleic acid stain DAPI was obtained from Sigma–Aldrich (St Louis, MO). Secondary antibodies coupled to HRP were obtained from Amersham Bioscience (Uppsala, Sweden) and fluorescent secondary antibodies coupled to Alexa fluorochromes were from Life Technology Corporation (Carlsbad, CA). Fluorescent mounting medium was from DAKO (Agilent Technologies, Santa Clara, CA, USA).

2.2. Cells lines and cell culture

Chinese hamster ovary (CHO) cell lines were grown in Ham's F-12 medium. The medium was supplemented with 10% of fetal bovine serum (FBS) and penicillin–streptomycin solution (10 units-10 μg). All cell cultures were maintained at 37 °C in a humidified atmosphere (5% CO_2).

2.3. Plasmids, site-directed mutagenesis and cloning

GCN4 leucine zipper split-luciferase constructs Zip-hGLuc1 and Zip-hGLuc2 in pcDNA3.1 vectors were obtained from the group of S.W. Michnick [37]. All the constructs expressing APP and APP fragments fused to humanized *Gaussia* luciferase (hGluc) halves were obtained by PCR amplification of APP sequences encoded by expression vectors previously described [19] with forward and reverse primers harboring the *NotI* and *ClaI* restriction sites, respectively. PCR products were digested and further inserted in the *NotI*/*ClaI* restrictions sites of the Zip-hGLuc1 and Zip-hGLuc2 constructs, removing the GCN4 leucine zipper sequence of the backbone. All constructs were verified by full sequencing (Macrogen Europe, Amsterdam, The Netherlands). C83 mutants were obtained by Quick-change site-specific mutagenesis (Stratagene, La Jolla, CA) as previously described [31].

2.4. Cell transfection and treatment

CHO cells were transfected with Lipofectamin reagent 24 h after seeding following manufacturer's instructions. Plasmids expressing the split-luciferase proteins were cotransfected in a 1:1 ratio. The control plasmid (Mock) was the corresponding empty vector. MEF cells (PS+/+ and PS−/−) were transfected using Trans-IT2020 according to the manufacturer's instructions. CHO cells were treated with DAPT for 15 h at a final concentration of 1 μM. 48 h after transfection, medium was collected, treated with protease inhibitors cocktail (Roche) and stored at 20 °C for ECLIA assay. Cells were harvested and lysed in Luciferase Cell Lysis Buffer (New England Biolab) and pelleted by quick centrifugation at 4 °C for 1 min. Protein concentrations of cell lysates were measured by the BCA protein assay kit (Pierce). Cell lysates were further used for *Gaussia* luciferase assay and Western blotting.