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Membrane tethering of APP c-terminal fragments is a prerequisite for T668 phosphorylation preventing nuclear sphere generation☆

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ABSTRACT

A central molecular hallmark of Alzheimer's disease (AD) is the β- and γ-secretase-mediated cleavage of the amyloid precursor protein (APP), which causes the generation of different c-terminal fragments like C99, AICD57, or AICD50 that fully or in part contain the APP transmembrane domain. In this study, we demonstrate that membrane-tethered C99 is phosphorylated by JNK3A at residue T668 (APP695 numbering) to a higher extent than AICD57, whereas AICD50 is not capable of being phosphorylated. The modification decreases the turnover of APP, while the blockade of APP cleavage increases APP phosphorylation. Generation of nuclear spheres, complexes consisting of the translocated AICD, FE65 and other proteins, is significantly reduced as soon as APP c-terminal fragments are accessible for phosphorylation. This APP modification, which we identified as significantly reduced in high plaque-load areas of the human brain, is linearly dependent on the level of APP expression. Accordingly, we show that APP abundance is likewise capable of modulating nuclear sphere generation. Thus, the precise and complex regulation of APP phosphorylation, abundance, and cleavage impacts the generation of nuclear spheres, which are under discussion of being of relevance in neurodegeneration and dementia. Future pharmacological manipulation of nuclear sphere generation may be a promising approach for AD treatment.

1. Introduction

The amyloid precursor protein (APP) undergoes amyloidogenic processing by the β-secretase to generate the extracellular fragment sAPPβ and a c-terminal transmembrane-fragment (CTF) of 99aa in length (C99). The latter is further cleaved by the γ-secretase complex, which causes generation of the toxic β-amyloid peptide, i.e. the main constituent of amyloidogenic plaques that correspond to a central hallmark of Alzheimer's disease (AD). In addition, the intracellular APP domain (AICD), which has been related to cell toxicity, is another product of this amyloidogenic (as well as of the non-amyloidogenic) APP cleavage [1–3]. Like for β-amyloid, several AICD isoforms have been described, including (the putatively most important isoforms) AICD57/59 [4], AICD50 [5], and AICD31 [6]. Since the identification of AICD-dependent complexes in the nucleus [7], several studies in respect to the function, localization and relevance of this cleavage product have been undertaken. Ectopically expressed AICD57/59 was shown to be present in the cytoplasm and in the nucleus in MDCK cells [4], in the nucleus of H4 neuroglioma cells [8], and suggested to contribute to the activation of the p53-mediated apoptotic pathway [9]. In wildtype and mutant APP overexpressing CHO cells, the shorter AICD51/50 was identified [5,10], and this fragment was suggested to disrupt actin dynamics and mitochondrial bioenergetics, together with its interacting protein FE65 [11]. AICD31, the caspase-8/9-cleaved c-terminal APP fragment, was shown to exert neurotoxicity [12] and to be present in AD brains but not in controls [13]. Furthermore, it selectively increases β-amyloid-42 species [14]. In contrast, other reports reveal that AICD generated from the α-secretase-cleaved APP fragment C83 remains membrane-tethered in CHO cells and does not result in increased nuclear translocation [15]. All things considered, our present knowledge on the localization of different AICD isoforms is limited, and the conditions for nuclear translocation of these fragments are understood only in part.

Phosphorylation of APP at residue threonine 668 (T668, according to APP695 numbering) was suggested as one mechanism which may regulate the nuclear translocation of AICD [3]. T668 phosphorylation, which was reported to be undertaken by Cdk5 [16] and JNK3 [17], causes a conformational switch of AICD [18] to alter the specificity and affinity of binding to cytosolic proteins. Some studies suggest that the specific APP phosphorylation elevates the production of β-amyloid [19–21], but others found no correlation between β-amyloid levels and the APP phosphorylation state [22]. In addition, GSK3β-mediated

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APP phosphorylation has been shown to increase the stability of the precursor and to impede APP turnover [23], giving rise to the controversial discussion of APP T668 phosphorylation in APP metabolism. With regard to the situation in the human brain, two different studies have been conducted to date. Increased phosphorylated APP T668 levels were found in hippocampal lysates from 14 AD but not in 10 control samples [20]. Similar results were obtained using immunohistochemistry in hippocampal pyramidal neurons, neurons of the dentate gyrus, and ectorhinal cortex of AD brains [3], pointing to the relevance of this specific phosphorylation for the disease.

A central functional role for AICD (respectively CTFs), which has been controversially discussed for about 15 years [15,24], is its nuclear translocation and generation of protein complexes [7], which have also been termed nuclear spheres [25]. Proteins that have been identified in these nuclear aggregates, besides AICD, are the APP binding and adapter protein FE65, the histone acetyl-transferase TIP60, Ran-binding protein9 (RanBP9) [26] and the helicase BLM. The complexes may be involved in a variety of mechanisms, those including gene expression changes [12,27], DNA repair [28], and cell cycle re-entry [25]. Accordingly, studying nuclear sphere generation is important in order to unravel a putatively additional (beside β-amyloid generation) APP-dependent toxic signal transduction pathway. As APP T668 phosphorylation has been suggested to modulate the subcellular localization of APP cleavage fragments, this post-translational modification of APP may also impact the generation of nuclear spheres. However, the different c-terminal cleavage products, some of which are membrane-tethered while others are not, may be phosphorylated to a different extent as a consequence of their subcellular localization. Hence, the generation of nuclear spheres may depend on the generation of a specific CTF and its phosphorylation state.

2. Materials and methods

2.1.1. Human tissue preparation

Formalin-fixed, paraffin-embedded (FFPE) human cortical brain tissue samples, extracted post-mortem from Alzheimer's disease (AD, n = 6) patients, were kindly provided by the Institute of Neuropathology at the Universitätsklinikum in Münster. PE tissue sections (5 μm) were cut with a microtome (RM 2155 Microtome, Leica). For immunoblotting, 100 mg of brain tissue sample was dounce homogenized in DIGE buffer, after centrifugation at 16,000 × g, the supernatant was collected and the protein concentration was determined by using Bradford assay. An ethical vote for the work with human brain samples is available (no. 2875, ethical commission at Ruhr-University Bochum).

2.1.2. Immunohistochemistry

FFPE sections were deparaffinized in xylene and rehydrated in decreasing grades of ethanol. Additionally, the sections were incubated in 88% formic acid for 3 min. Heat induced antigen retrieval was performed at 100 °C in sodium citrate buffer for 40 min. The tissue was incubated with antibodies against phospho APP (T668) (1:100, GeneTex) and amyloid beta (1:500, 4G8, Convance). Amyloid beta was detected by a HRP-coupled anti-mouse (1:500, Jackson Immuno Research) secondary antibody, while phosphor APP (T668) was visualized using the ImmunoCruz™ rabbit ABC Staining System (Santa Cruz, sc-2018). Nuclear visualization was carried out by hematoxylin counterstaining. The sections were mounted in antifade medium (Fluorescence Mounting Medium, Dako) on cover slips.

2.1.3. Quantification

The ratio of the total cell number and phospho APP (T668)-positive cells was determined. Using the 40× lens of the IX51 microscope (Olympus), 15 plaque-positive and plaque-negative areas per sample were chosen and the number of total cells, and phospho APP-positive stained cells in each of the areas were counted.

2.1.4. Cell culture, transfections and microscopy

HEK293, HEK293T and SH-SY5Y cells were grown and maintained in DMEM media (Invitrogen) with 10% FBS and 5% penicillin/streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Prior to be used in experiments, all cell lines were found to be mycoplasma free with the aid of mycoplasma detection kit (Roche, Germany). Cell culture reagents were purchased from life technologies. For all transfections conditions, cells were grown till 70% confluency and then transfected. Transient transfections were performed using an optimized polyethylenimine (PEI) transfection system, described in our previous publication [29]. For the immunoblotting experiments, 3×10^6 HEK-293 T cells were seeded in 10 cm cell culture plates, while for counting of spheres positive cells experiments, the cells were seeded at a density of 50,000 cells/well in a 24-well cell culture plate. For transfections, 12 μg of DNA were used for subsequent immunoblotting experiments, while 2 μg/well of DNA were used for sphere positive cells counting experiments. The amount of DNA used for different constructs in different transfections was balanced by using the empty vector pcDNA3.1. For microscopy, cells were fixed using 4% PFA after 24 h of transfection. Images were taken with SLR camera (Olympus) and Cell P software (Olympus) on a fluorescence microscope (IX50, Olympus, Germany).

For confocal imaging, HEK293T cells were seeded in ibiTreat-coated μ-Slide 8 Well from ibidi® (Martinsried, DE) and 24 h after seeding transfected using Polyethylenimine (PEI) from Sigma (Taufkirchen, DE). 24 h after transfection the cells were fixed with Roti®-Histofix 4% (4% phosphate buffered formaldehyde solution; Roth, Karlsruhe, DE) for 20 min at RT and permeabilized with 0.3% (v/v) Triton X-100 in PBS for 10 min at RT. The DNA was stained with 1 μg/ml Hoechst3334 dye in PBS for 10 min at RT. 0.1% (w/v) sodium azide in PBS was added to the samples in order to prevent the growth of microbial organisms. Samples were imaged with a Leica SP8 confocal microscope equipped with a HCX PL Apo CS 63.0 × 1.20 water UV objective, a sensitive hybrid detector and the following lasers: Diode 405, Argon and DPSS561. Fluorophores were excited with 405/488/514/561 nm laser lines performing a sequential scan beginning with the longest wavelength. Images were recorded with a resolution of 1024 × 1024 pixels at a scan speed of 200 Hz.

2.1.5. Plasmid constructs

The source of plasmid constructs pEGFP-N1-AICD50, pEGFP-N1-AICD57, 2N3T-TIP60, wtAPP-YFP, FE65-EGFP and pcDNA3 Flag MKK7B2JNK3a can be found in our previous publications [30]. The pcDNA4 BACE1 mycHis vector was a kind gift from Weihong Song [31]. The C99-YFP was amplified from the FL-APP vector using the KLD mutation kit (NEB) in accordance with manufacturer's instructions. The FE65-mCherry-N1 vector was constructed by inserting the FE65 sequence into the mCherry2-N1 vector with the aid of NEBuilder® HiFi DNA Assembly Master Mix (NEB) according to the manufacturer's instructions. The primers sequence used for the generation of C99-YFP and FE65-mCherry-N1 vectors can be provided upon request.

2.1.6. Generation of APP stable cell lines and inhibitor treatment

For the generation of APP-YFP stable cell lines, both neuronal SH-SY5Y and non-neuronal HEK293 cell lines were used. 3×0^6 cells were seeded in 10 cm cell culture plates and were transfected with the wtAPP-YFP construct using the PEI transfection method. Since SH-SY5Y cells cannot be readily transfected, the transfection duration was extended to 24 h, while it was 4 h for HEK293 cell. After 24 h of transfection, the transfected cells were selected, and untransfected cells were eluded by using 1000 μg/ml of Geneticin (G418). Selected clones were