Neuroscience Letters 521 (2012) 82–87

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

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

Immunoreactivity of the amino-terminal portion of the amyloid-beta precursor protein in the nucleolus

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HIGHLIGHTS

- The N-terminal portion of APP localizes in the nucleus and the nucleolus.
- The GS4463 antibody co-localizes with APP and fibrillarin, the nucleolus marker.
- These findings indicate the function of APP in the nucleolus.

ARTICLE INFO

Article history:
Received 7 March 2012
Received in revised form 18 May 2012
Accepted 22 May 2012

Keywords:
Alzheimer's disease
Amyloid-β A4 precursor protein
Calcium signaling
GM1
Nuclear translocation
Nucleolus
rRNA

ABSTRACT

The functioning and metabolic pathway of the amyloid β-precursor protein (APP) have not been fully elucidated. To fill this research gap, this study immunocytochemically investigated the intracellular localization of APP in the neuroblastoma cell line SK-N-SH and in normal primary cells. Using antibodies against the amino-terminal portion of the APP molecule, immunoreactivity was detected not only in the cytoplasm but also in the nucleus and nucleolus. Further analysis revealed the co-localization of amino acids 44–63 of the APP molecule with fibrillarin, a nucleolus marker. These findings indicate that a fraction of APP, including its amino-terminal portion, may be localized in the nucleus as well as in the nucleolus, suggesting an important role of APP in RNA metabolism and other intra-nucleolus functions.

1. Introduction

The amyloid-β A4 precursor protein (APP) is a precursor of amyloid β (Aβ), which is closely associated with the pathology of Alzheimer's disease [14] and is a type I single-pass transmembrane protein of 695, 751, or 770 amino acids of splicing isoforms [8]. It is commonly accepted that the subcellular localization of APP occurs predominantly in the Golgi apparatus and the endoplasmic reticulum [22].

Several studies have reported nuclear translocation of the cytoplasmic tail of APP, referred to as the APP intracellular domain (AICD). Based on the investigation of the AICD using forced AICD-fusion expression and co-expression techniques, several researchers have suggested that the AICD forms a multi-protein complex with Fe65 and Tip60 in the nucleus [11,22,23]. However, these results have not contradicted the possibility of translocation of the full-length APP molecule, including the AICD, naturally expressed in the cell.

The possibility of such translocation must not only be investigated but the primary role of APP and its explicit metabolic pathway must also be defined. Using immunofluorescence microscopy to examine the N-terminal portion of the molecule, this study investigates the intracellular localization of native APP in a neuroblastoma cell and in normal primary cells.

2. Materials and methods

2.1. Antibodies and peptide

The primary antibodies used were (1) APP antibody (44–63), rabbit pAb (No. A00692; Genscript, Piscataway, NJ, USA), referred to as GS4463 in this study; (2) anti-human APP (N) (10D1; No. 11090 IBL Co., Ltd. Tokyo, Japan), referred to as 10D1; (3) anti-Alzheimer precursor protein A4, a.a. 66–81 of APP {N-terminus}, clone 22C11 No. MAB348, mouse IgG monoclonal antibody (Millipore, Billerica, MA, USA), referred to as 22C11; (4) fibrillarin antibody [38F3] (No. ab4566; Abcam, Cambridge, MA, USA); (5) eIF6

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http://dx.doi.org/10.1016/j.neulet.2012.05.064

[D16E9] rabbit mAb (#3833S; Cell Signaling Technology, Boston, MA, USA); and (6) anti-GAPDH rabbit monoclonal antibody 14C10 (No. 2118; Cell Signaling Technology). The secondary antibodies used for immunofluorostaining were (1) Alexa FluorR 488 (No. A11001) goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), (2) Alexa FluorR 488 (No. A11008) goat anti-rabbit IgG (H+L; Invitrogen), and (3) Alexa FluorR 555 (No. A21422) goat anti-mouse IgG (H+L; Invitrogen). The antibodies used for western blotting were (1) rabbit polyclonal anti-mouse immunoglobulin alkaline phosphatase conjugates (No. D0314; DAKO, Glostrup, Denmark) and (2) goat polyclonal anti-rabbit immunoglobulin alkaline phosphatase conjugates (No. D0487; DAKO). Ac-HMNVQNGKWDSDPSGTKTCI-CONH2, a peptide corresponding to the N-terminal amino acid 44–63 of human APP and of 92% purity, referred to HMN20 in this study, was obtained from Operon Biotechnologies (Tokyo, Japan).

2.2. Immunofluorescence microscopy

A human neuroblastoma cell line SK-N-SH was cultured in MEM-α with 10% FBS. Normal human asctrocytes and human renal epithelial cells were cultured in each dedicated medium at 37 °C and 5% CO_2. Procedures for immunostaining were followed from the guidelines by Abcam and performed at 0 °C on ice [9]. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.25% Triton X-100 in PBS and blocked with 5% goat serum in PBS. The primary antibodies were used at 1 to 5 μg/ml and Alexa conjugates (Invitrogen) were used as secondary antibodies at 1:1000 dilution. The cells were mounted with antifade reagent containing DAPI and imaged using Carl Zeiss LSM 510 META Confocal Microscope.

2.3. Fractionation of cell extracts and Western blotting analysis

Cell extracts were fractionated using the method described by Schreiber et al. [20]. Ten micrograms of proteins, after SDS-polyacrylamide gel electrophoresis, were blotted to a nitrocellulose membrane using iBlot system (Invitrogen) and analyzed according to the manufacturer's instructions.

3. Results

3.1. Anti-APP N-terminal antibody staining of SK-N-SH cell nuclei

A schematic diagram of the epitopes of the antibodies used to examine the intracellular localization of APP in the human neuroblastoma cell line SK-N-SH is shown in Fig. 1A. Both 10D1 and GS4463 were found to stain the cytoplasm and the entire nucleus of the cells, with the GS4463 staining showing a punctuated staining pattern in the inner nucleus with a nucleolus-like or speckle-like structure. In contrast, the 22C11 had web-like staining pattern primarily observed in the cytoplasm, and was thus relatively different from that of the other 2 antibodies. The cytoplasmic and nuclear staining with GS4463 antibody was not observed when it was preincubated with 50 μg/ml of the synthetic peptide of amino acid residue 44–63 of APP, indicating that the signal had resulted from the specific recognition of 44–63 amino acid sequence of APP.

3.2. Co-localization of GS4463 signals with nucleolus markers in SK-N-SH cells

To examine the intra-nuclear structure stained by GS4463, SK-N-SH cells were stained by antibodies against the nucleolus markers, fibrillarin and eIF6. GS4463 signals completely co-localized with the fibrillarin and eIF6 signals, confirming the co-localization of APP and nucleolus markers (Fig. 2). However, GS4463 did not co-localize with the speckle marker SC35 antibody (ab11826; data not shown), suggesting that a fraction of APP is located in the nucleolus of the cultured neuroblastoma cell line SK-N-SH.

3.3. GS4463 staining of the nucleolus of primary cultured cells

Further, APP localization in the primary cultured cells from normal human brain and kidney tissue was examined using 10D1 and GS4463. 10D1 stained the nucleus of HRECs similar to the SK-N-SH cells, but intensely stained the cytoplasm in NHAs (Fig. 3). GS4463 stained the nucleolus in both NHAs and HRECs similar to that in SK-N-SH cells (Fig. 3). These signals were not observed when the antibodies were pre-incubated with the synthetic peptide amino acid 44–63 residues of APP, suggesting localization of an APP fraction in the nucleolus of normal brain and kidney cells.

3.4. GS4463 reactivity in nuclear extracts

Cytoplasmic and nuclear extracts of SK-N-SH cells cultured in MEM-α were analyzed by western blotting to examine the distribution and molecular sizes of the APP-processing products in each fraction. Successful enrichment of the cell extracts from each fraction was confirmed using GAPDH and fibrillarin antibodies (Fig. 4A). A band of 110–120 kD, believed to indicate full-length APP, was observed in the cytoplasmic fraction when stained with all 3 antibodies. Additional higher molecular weight bands with a smeared pattern, such as those indicating glycosylation, were observed by 10D1 and 22C11 staining, while the nuclear-fraction specific 30 kD band was observed by 10D1 staining. No major band was observed in the nuclear extracts by 22C11 staining.

In addition, a 50 kD, a 110 kD, and a 120 kD product were observed in the cytoplasm by GS4463 staining, whereas this 110 kD

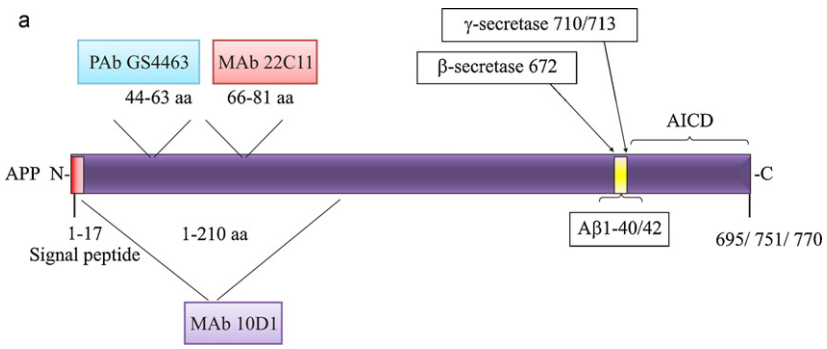

Fig. 1. (A) Schematic representation of epitopes of the antibodies used in study and known amyloidogenic-processing sites of APP. (B) Immunofluorescence microscopy of anti-APP. Human neuroblastoma SK-N-SH cells cultured in αMEM were immunostained by the indicated antibodies as described. In the antigen competition experiment, anti-APP antibody, GS4463 was preincubated with 100 μg/ml of HMN20 peptide (the bottom right panel).