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Research paper

Phosphorylation of Fe65 amyloid precursor protein-binding protein in response to neuronal differentiation

Niina A. Koistinen, Smaranda Bacanu, Kerstin Iverfeldt*

Stockholm University, Department of Neurochemistry, 10691 Stockholm, Sweden

HIGHLIGHTS

- RA, PMA, and DAPT induce an electrophoretic mobility shift of Fe65 in SH-SY5Y cells.
- NGF induces an electrophoretic mobility shift of Fe65 in PC6.3 cells.
- The electrophoretic mobility shift of Fe65 was reversed by alkaline phosphatase.

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ABSTRACT

Fe65 is a brain enriched multi domain adaptor protein involved in diverse cellular functions. One of its binding partners is the amyloid-β (Aβ) precursor protein (APP), which after sequential proteolytic processing by secretases gives rise to the Alzheimer's Aβ peptide. Fe65 binds to the APP intracellular domain (AICD). Several studies have indicated that Fe65 binding promotes the amyloidogenic processing of APP. It has previously been shown that expression of APP increases concomitantly with a shift of its processing to the non-amyloidogenic pathway during neuronal differentiation. In this study we wanted to investigate the effects of neuronal differentiation on Fe65 expression. We observed that differentiation of SH-SY5Y human neuroblastoma cells induced by retinoic acid (RA), the phorbol ester PMA, or the γ-secretase inhibitor DAPT resulted in an electrophoretic mobility shift of Fe65. Similar effects were observed in rat PC6.3 cells treated with nerve growth factor. The electrophoretic mobility shift was shown to be due to phosphorylation. Previous studies have shown that Fe65 phosphorylation can prevent the APP-Fe65 interaction. We propose that phosphorylation is a way to modify the functions of Fe65 and to promote the non-amyloidogenic processing of APP during neuronal differentiation.

1. Introduction

Fe65 is a neuronal cytoplasmic adaptor protein consisting of three well-conserved protein–protein interaction domains: a tryptophan–tryptophan (WW) domain and two adjacent phosphotyrosine binding (PTB) domains, PTB1 and PTB2 [24,32,33]. Through these domains Fe65 can bind to various other proteins forming multimeric complexes involved in diverse cellular functions. One Fe65 binding protein of particular interest in the field of Alzheimer's disease (AD) is the amyloid-β precursor protein (APP). Fe65 has been reported to regulate secretase-mediated processing of APP and hence the production of the neurotoxic amyloid β (Aβ) peptide [35,37,39]. Abnormal production or insufficient removal followed by aggregation of Aβ in the brain is believed to be a key pathogenic event leading to neuronal cell death in AD (reviewed in [27]). Fe65 interacts with the YENPTY motif of APP intracellular domain (AICD) [4]. It has been shown that AICD, released by γ-secretase cleavage, translocates into the nucleus in a process that involves binding to the Fe65 PTB2 domain [19,20,33]. Fe65 was early shown to possess transcriptional activity [10]. In fact, AICD together with Fe65 has been reported to be involved in regulating the transcription of multiple target genes [2,18,30,38]. The interaction between APP and Fe65 has further been shown to be regulated by phosphoryla-

Abbreviations: Aβ, amyloid-β; AD, Alzheimers' disease; AICD, APP intracellular domain; APP, amyloid precursor protein; ATM/ATR, ataxia telangiectasia mutated/ataxia-telangiectasia-and Rad3-related; BCA, bicinchoninic acid; CHO, Chinese hamster ovary; CPI, complete protease inhibitor; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; ECL, enhanced chemiluminescence; ERK1/2, extracellular-signal-regulated kinase 1/2; NGF, nerve growth factor; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, 12-myristate 13-acetate; PMSF, phenylmethanesulfonylfluoride; PTB, phosphotyrosine binding; PVDF, polyvinylidine difluoride; RA, retinoic acid; SGK1, serum-and glucocorticoid-induced kinase 1; WW, tryptophan tryptophan.

* Corresponding author. Fax: +46 8 161371.
E-mail address: kerstin@neurochem.su.se (K. Iverfeldt).

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tion. Phosphorylation within AICD (at Thr668) reduces the affinity of APP to Fe65 [1]. Recent studies have also shown functional effects mediated by phosphorylation of Fe65 itself. Tyr or Ser phosphorylation within the PTB2 domain was shown to enhance Fe65-APP mediated transcription or to promote translocation of Fe65 to the nucleus [8,22,31].

Both APP and Fe65 are present in growth cones and synapses [34], and have been implicated in neuronal development. Expression of the neuron enriched isoform of APP (APP695) increases during neuronal differentiation [15,21]. The expression of Fe65 has been shown to be developmentally regulated in mouse [17], and increases postnatally in a similar way as observed for APP in rat brain [23]. Our previous studies showed that retinoic acid (RA)-induced neuronal differentiation of human SH-SY5Y neuroblastoma cells leads to increased expression levels of APP and APP processing enzymes [11], as well as increased non-amyloidogenic processing of APP [12]. Due to the proposed role of Fe65 in APP metabolism and transcriptional activity we wanted to further investigate the effect of neuronal differentiation on Fe65 expression. In addition to RA, a phorbol ester (PMA), which also induces neuronal differentiation of SH-SY5 Y cells [28,29], was used. The cells were also exposed to the γ-secretase inhibitor DAPT that has previously been shown to have a differentiating effect on several stem cell lines through the inhibition of Notch activity [5,9]. Here, we demonstrate that neuronal differentiation of SH-SY5Y neuroblastoma cells induces an electrophoretic mobility shift of endogenous Fe65 and that this shift is due to increased Fe65 phosphorylation.

2. Materials and methods

2.1. Cell culture and treatment

SH-SY5Y human neuroblastoma cells (American Type Culture Collection) were routinely maintained in minimum essential medium with Earl's salts, 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, and 1% penicillin-streptomycin. All reagents were purchased from Life Technologies. The cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 50,000 cells/cm^2. 24 h after seeding, cells were grown in the absence or presence of 10 μM all-*trans* retinoic acid (RA, Sigma), or 16 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 3 days. For studies on γ-secretase inhibition, 5 μM *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine t-butyl ester (DAPT, Sigma) was added 48 h after seeding cells and 24 h prior to harvesting. MTT cell viability assay (using the reagent M5655, Sigma) did not indicate any cytotoxic effect of DAPT (less than 5% effect on cell viability for 5, 10, and 20 μM).

2.1.1. siRNA knockdown

For Fe65 knock-down studies, cells were transfected with 50 nM of FlexiTube siRNA (Qiagen; Hs_APBB1_8) for 48 h using Lipofectamine® RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol.

2.1.2. Harvesting of cells and western blot assay

Cells from each treatment were washed with 2 × 4 ml of ice-cold 1 × phosphate buffered saline (PBS) and lysed for 30 min at 4 °C in RIPA-buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)) supplemented with complete protease inhibitor (CPI) cocktail (Roche). The cell lysates were centrifuged at 10,000 × *g* for 5 min at 4 °C before the total protein concentration of each sample was measured using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc.,) according to manufacturer's protocol. For detection of Fe65, cell lysate, corresponding to 10 μg protein, was subjected to electrophoresis on an 8% polyacrylamide gel. Proteins were subsequently transferred to a polyvinylidine difluoride (PVDF) or nitrocellulose membranes (GE Healthcare) for 1 h at a current of 500 mA. Non-specific binding to the membranes were blocked by incubation with 5% non-fat dry milk in prior to incubation with a primary antibody over night at 4 °C. The membranes were subject to a secondary antibody for 30 min at room tem-

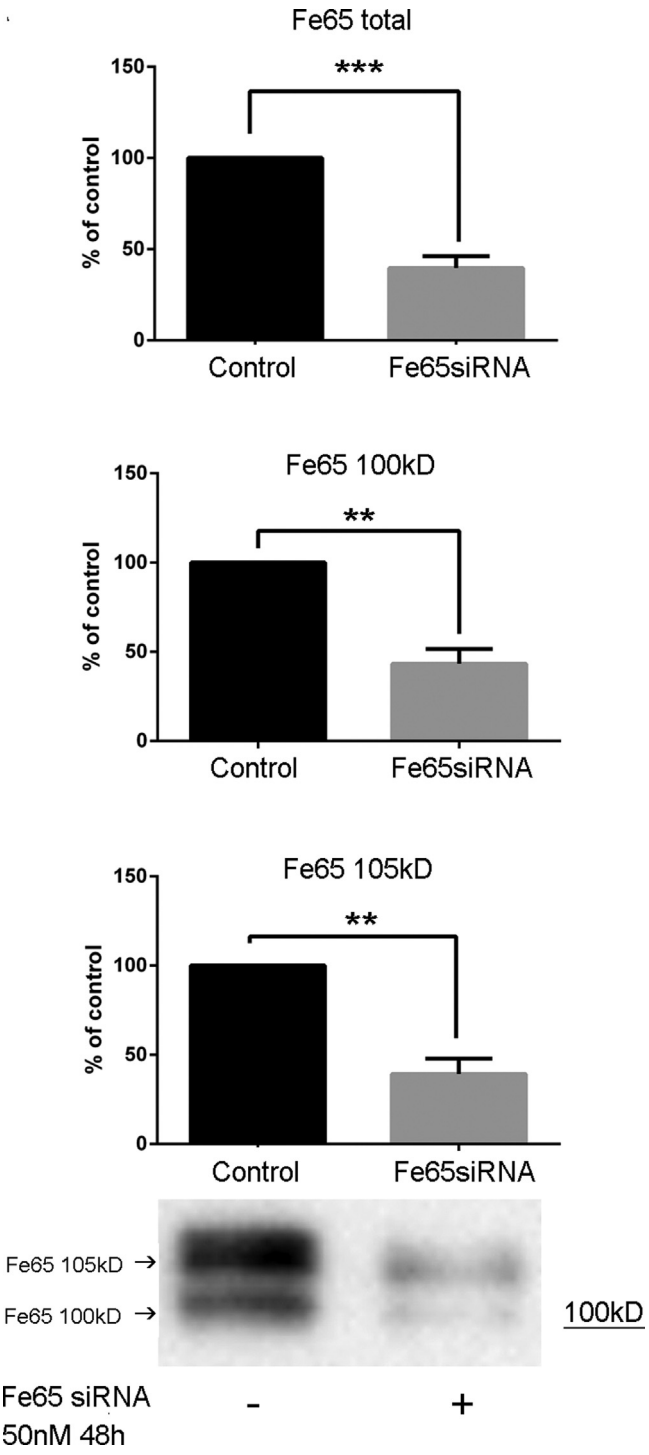

Fig. 1. Fe65 migrates as several bands on western blot with apparent molecular weight around 100 kDa and 105 kDa. Relative abundance of Fe65 total (100 kDa + 105 kDa), Fe65 100 kDa and Fe65 105 kDa in cell lysates after transfection with 50 nM Fe65 siRNA. Representative western blot analysis of Fe65 is shown below the graphs. Data represents mean ± S.E., $n = 3$, ** $p < 0.01$ and *** $p < 0.001$ significantly different from control.