Roles of the intramolecular regions of FE65 in its trans-accumulation and in p53 stabilization in the nuclear matrix of osmotically stressed cells

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ABSTRACT

The neural adaptor protein FE65 interacts with the amyloid β-protein precursor (APP). In osmotically stressed cells, the membrane APP-tethered FE65 is released into the cytoplasm and translocates to the nuclear matrix, where it stabilizes p53 via a non-canonical pathway. In this study, we found that the second phosphotyrosine interaction domain (PI2) of FE65 mediated its trans-accumulation in the nuclear matrix of osmotically stressed cells. The carboxyl-terminal half of FE65, which contains the PI2 domain, failed to stabilize p53, suggesting that the amino-terminal half of the protein plays an important role in the stabilization of p53 in osmotically stressed cells.

1. Introduction

FE65 is a cytoplasmic adaptor protein that interacts with various proteins [1], including the amyloid β-protein precursor (APP), which is a causative factor of Alzheimer’s disease. FE65 is thought to function in the regulation of various cellular events by interacting with these proteins. Although FE65 knockout mice exhibit impairment of learning and memory abilities [2] and an abnormality in brain structure [3], the precise physiological function of FE65 remains unclear.

A recent biochemical analysis of FE65 suggests that this protein is involved in cell stress and/or DNA damage [4]. A fixed population of cytoplasmic FE65 is tethered to the membrane by binding to APP. In turn, the tethered FE65 is released into the cytosol via the phosphorylation of APP in hyperosmotically stressed cells [5–7]. The released FE65 then trans-accumulates in the nuclear matrix and participates in DNA damage response by inducing γH2AX and by stabilizing p53 [7,8]. FE65 comprises one WW domain, which is composed of two conserved tryptophan (W) residues spaced 20–22 residues apart within the consensus-sequence-containing domain [9], and binds to a proline-rich sequence and two phosphotyrosine interacting domains (PI1 and PI2); however, the roles of these domains in the nuclear-matrix accumulation of FE65 and in the stabilization of p53 in osmotically stressed cells remains unknown. In this study, we identified the intramolecular regions of FE65 required for the nuclear-matrix trans-accumulation of FE65 and for the stabilization of p53 therein in hyperosmotically stressed cells.

2. Materials and methods

2.1. Antibodies and plasmids

The rabbit polyclonal anti-enhanced green fluorescent protein (EGFP) (598B, MBL, Japan), mouse monoclonal anti-actin (C4, Chemicon/Millipore, USA), and anti-FLAG (M2, Sigma/Aldrich, USA) antibodies were purchased. To prepare FE65-deletion mutants fused to EGFP, the cDNA of FE65 [7] was amplified by PCR using specific primers. The PCR products were digested with KpnI/BamHI and cloned into the pEGFP-N3 vector (Clontech/Life Technologies, USA) to generate pEGFP-N, pEGFP-C, pEGFP-ΔPI1, pEGFP-ΔPI2, pEGFP-ΔPI1ΔPI2, and pEGFP-PI2. For FLAG-tagged constructs, the PCR products were digested with NheI/NotI and cloned into the pcDNA3.1NFLAG vector [6] to generate pcDNA3.1NFLAG-FE65N and pcDNA3.1NFLAG-FE65C. pcDNA3.1NFLAG-FE65full and pcDNA3.1NFLAG-p53 were as described previously [6,8].
2.2. Cell culture, plasmid transfection, sorbitol treatment, subnuclear fractionation, and immunoblotting of proteins

A mouse Neuro2a cell line was used throughout this study. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen/Life Technologies, USA). For sorbitol treatment, cells were treated with 0.5 M sorbitol for 45 min. For subnuclear fractionation, proteins were extracted consecutively using buffers that were adequate to obtain membrane/cytosol (MC), nucleoplasm (NP), DNA-binding proteins (DNA-bp), RNA-binding proteins (RNA-bp), and nuclear-matrix (NM) fractions [7]. For immunoblotting of proteins, proteins were separated by SDS–PAGE, transferred onto a nitrocellulose membrane, and were then incubated with the primary antibody. After incubation with the secondary antibody, i.e., anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare Lifescience, USA), immunoreactive proteins were detected by ECL (GE Healthcare) and were quantified using the luminescent image analyzer LAS-4000mini (Fujifilm, Japan). The proteins detected using the anti-EGFP antibody were standardized to actin levels, which were assessed using an anti-actin antibody.

2.3. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

MTT (10 μL of a 5 mg/mL solution; Sigma/Aldrich) was added to the cells for a period of 6 h. The reaction was halted by the addition of 100 μL of 0.04 N HCl in isopropanol and optical density at 570 nm (with a reference at 630 nm) was measured using a microplate reader.

3. Results

3.1. Role of the N- and C-terminal regions of FE65 in its nuclear-matrix trans-accumulation and in the suppression of the reduction of cell viability in osmotically stressed cells

To identify the domains of FE65 required for its nuclear-matrix trans-accumulation in osmotically stressed cells, we first examined the N-terminal (FE65N, 1–368) and C-terminal (FE65C, 369–710) regions of FE65 tagged with EGFP at their C termini (Fig. 1A). Cells expressing FE65 were treated with (+) or without (−) sorbitol for 45 min and the cell lysates were fractionated into crude lysate (CL), MC, NP, DNA-bp, RNA-bp, and NM, as described previously [7]. The distribution of the proteins was analyzed using immunoblotting (Fig. 1B), which revealed recovery of FE65 proteins in the NM fraction (Fig. 1C). EGFP-full was largely recovered in the MC fraction, regardless of sorbitol treatment, and approximately 20% of EGFP-full was recovered in the NM fraction after sorbitol treatment (Fig. 1B and panel a of Fig. 1C), as was observed for endogenous FE65 [7]. This result suggests that EGFP tagging had no effect on the localization of the protein. EGFP-N was largely recovered in the MC fraction, as was EGFP-full; however, almost no protein was recovered in the NM fraction (Fig. 1B and panel b in Fig. 1C).

In sorbitol-treated cells, the recovery of EGFP-N in the NM fraction was much lower than that observed for EGFP-full.

In contrast to EGFP-N, EGFP-C showed a distribution that was similar to that of EGFP-full (Fig. 1B and panel c of Fig. 1C). We assessed the exact net recovery of FE65 in NM fractions of cells after sorbitol treatment by subtracting the amount of FE65 detected in the NM fraction of cells prior to the treatment (panel d of Fig. 1C). The net trans-accumulation of EGFP-C was slightly increased in the NM fraction after sorbitol treatment (recovery, 23.4 ± 2.6%), while that of EGFP-N was markedly decreased (recovery, 1.5 ± 1.1%), when compared with EGFP-full (recovery, 16.2 ± 2%). These results indicate clearly that the nuclear matrix trans-accumulation of FE65 in osmotically stressed cells was dependent on its C-terminal domain.

Knockdown of FE65 increases apoptosis in cells treated with sorbitol over a long period [7], which suggests that FE65 plays a role in cell viability. Therefore, we assayed the viability of cells expressing FE65 after long-term sorbitol treatment (Fig. 1D). Cells with or without (−) expression of EGFP-full, EGFP-N, or EGFP-C were treated with sorbitol for 24 h and cell viability was quantified using an MTT assay. Cells expressing either EGFP-full or EGFP-N retained their viability at levels that were similar to those of the control (−). In addition, approximately 70% of treated cells were viable compared with non-treated cells (100%), while cells expressing EGFP-C had a significantly reduced viability, similarly to what was observed in FE65 knockdown cells [7]. These results suggest that the C-terminal domain of FE65 was essential for the trans-accumulation of FE65 in the nuclear matrix and that its N-terminal domain prevented the reduction in the viability of cells after hyperosmotic stress. EGFP-C may behave as a dominant-negative molecule to endogenous FE65 and suppress the function of endogenous FE65 during the promotion of cell survival.

3.2. Role of the N-terminal region of FE65 in the stabilization of p53

FE65 stabilizes p53 in the nuclear matrix of osmotically stressed cells [8]. To identify the intramolecular region of FE65 that is responsible for this function, we examined p53 stability in cells expressing N-terminally FLAG-tagged full-length FE65 (NFLAG-FE65full), the N-terminal half of FE65 (NFLAG-FE65N), or the C-terminal half of FE65 (NFLAG-FE65C) (Fig. 2). Cells expressing the NFLAG-p53 and NFLAG-FE65 proteins were treated with (Fig. 2A, lanes 9–16) or without (lanes 1–8) sorbitol, and cell lysates were subjected to immunoblotting using anti-FLAG (upper panel) or anti-actin (lower panel) antibodies. The levels of expression of NFLAG-p53 were almost identical in the absence of sorbitol treatment, regardless of the nature of the NFLAG-FE65 proteins that were co-expressed (lanes 1–8). Treatment of these cells with sorbitol (lanes 9–16) led to the stabilization of NFLAG-p53 in cells that co-expressed NFLAG-FE65full, which was enhanced by the increase in the expression of NFLAG-FE65full (Fig. 2A, lanes 10–12, and Fig. 2B). In contrast, NFLAG-FE65N and NFLAG-FE65C failed to stabilize NFLAG-p53 in sorbitol-treated cells (Fig. 2A, lanes 13–16, and Fig. 2B). As observed in Fig. 1, NFLAG-FE65N failed to trans-accumulate in the nuclear matrix, while NFLAG-FE65C and NFLAG-FE65full accumulated in the nuclear matrix in sorbitol-treated cells (data not shown). These observations indicate that the N-terminal half of FE65 plays an important role in the stabilization of p53 in the nuclear matrix of osmotically stressed cells, although we cannot rule out the possibility that NFLAG-FE65C may require the N-terminal half of the protein to retain a structure that is suitable for its role in the stabilization of p53.

3.3. The P12 domain was required for the nuclear-matrix trans-accumulation of FE65

The P11 and P12 domains located in the C-terminal region of FE65 are candidate domains for the mediation of the trans-accumulation of FE65 in the nuclear matrix. To determine which domain plays this role, we generated the following deletion mutants of FE65: EGFP-ΔP11ΔP2, EGFP-ΔP11, and EGFP-ΔP12 (Fig. 3A). Cells expressing these mutant FE65 proteins were treated with sorbitol, and the trans-accumulation of the proteins in the nuclear matrix was examined (Fig. 3B and C). Interestingly, EGFP-ΔP12 and EGFP-ΔP11ΔP2 lost the ability to trans-accumulate in the nuclear matrix in cells treated with sorbitol, while EGFP-ΔP11 trans-accumulated successively in the nuclear matrix. The recovery of EGFP-ΔP11 in the nuclear matrix was approximately 30%
expressing the indicated proteins (EGFP-full, EGFP-N, and EGFP-C) or control cells (Fig. 3C). The trans-accumulation of EGFP-N (panel b of Fig. 3C), which was in agreement with the results observed for EGFP-C (Fig. 1C), while the recovery of EGFP-ΔPI1ΔPI2 (2.7 ± 1.4%) and EGFP-ΔPI2 (1.9 ± 1.1%) was extremely low (panels a and c of Fig. 3C). We confirmed this observation using EGFP-PI2 (Fig. 3A), which was detected in the NM fraction of cells treated with sorbitol with a recovery of 12.3 ± 3.4% (Fig. 3B and C). Although the trans-accumulation efficiency of EGFP-ΔPI2 was lower than that of EGFP-ΔPI1, this result confirmed that PI2 is the minimum region required for the trans-accumulation of FE65. We also assessed the exact net recovery of FE65 in NM fractions (panel e of Fig. 3C). The trans-accumulation of EGFP-ΔPI1ΔPI2 and EGFP-ΔPI2 was significantly decreased in the NM fraction of cells treated with sorbitol. EGFP-ΔPI1 and EGFP-ΔPI2 did not exhibit a significant difference when compared with the recovery of EGFP-full (for EGFP-full, see Fig. 1C). These results clearly indicate that the PI2 domain mediated the nuclear-matrix trans-accumulation of FE65.

4. Discussion

In this report, we described two functional regions of FE65 that play an important role in the trans-accumulation of p53 and stabilization of p53 in osmotically stressed cells.

Fig. 1. Role of the C-terminal domain of FE65 in its trans-accumulation in the nuclear matrix of hyperosmotically stressed cells. (A) Schematic structure of the FE65 proteins used in this study. The human FE65 (FE65) and EGFP-full, EGFP-N, and EGFP-C, which were tagged C-terminally with EGFP, are indicated, as are the WW (255–283), PI1 (369–509), and PI2 (529–661) domains. The numbers indicate amino-acid positions. (B) Subnuclear distribution of the FE65 proteins. Cells expressing the various FE65 proteins were treated with (+) or without (−) sorbitol and subjected to subnuclear fractionation. Proteins were detected by immunoblotting using anti-EGFP or anti-actin antibodies. CL, crude lysate; MC, membrane and cytosol; NP, nucleoplasm; DNA-bp, DNA-binding proteins; RNA-bp, RNA-binding proteins; NM, nuclear matrix. (C) Recovery of FE65 proteins in the nuclear matrix. The proteins (a, EGFP-full; b, EGFP-N; c, EGFP-C) in the CL and NM fractions of panel b were quantified and standardized to actin and then their recovery (%) in the NM fraction vs. total amount (100% in CL) is shown. Cells treated with (+) and without (−) sorbitol were compared. The net recovery (panel d) was calculated by subtracting the amount detected in the NM of cells treated without sorbitol from that of cells treated with sorbitol. Data represent the mean ± standard deviation (S.D.) (n = 3). *P < 0.05, **P < 0.005, as assessed using a Student’s t-test against the control (−).

Fig. 2. Role of the N-terminal region of FE65 in the stabilization of p53. (A) Effects of the FE65 mutants in the stabilization of p53 in osmotically stressed cells. Cells expressing NFLAG-p53 and NFLAG-Fe65full (full), NFLAG-Fe65N (N), or NFLAG-Fe65C (C) were treated with (lanes 9–16) or without (lanes 1–8) sorbitol. (+) Cells transfected with the indicated plasmid; (−) cells transfected with vector alone. Numbers represent the amount (ng) of plasmids transfected. The cells were lysed and the cell lysates were subjected to immunoblotting using anti-FLAG (top) and anti-actin (bottom) antibodies. (B) Quantification of the levels of NFLAG-p53 in the nuclear matrix of the sorbitol-treated cells shown in panel a. The protein band intensities of NFLAG-p53 in sorbitol-treated cells (A, lanes 10, 12, 14, and 16) were quantified and normalized to the value of the control in panel a. The results represent the average of three experiments.

Error bar indicates the standard error. *P < 0.05, as assessed using a Student’s t-test.
First, the PI2 domain in the C-terminal half of the protein was required for the nuclear-matrix trans-accumulation of FE65 in osmotically stressed cells. Previous observations showed that (1) the PI2 domain interacts with the cytoplasmic NPXY (Asn-Pro-Thr-Tyr) motif (this motif induces a conformational change in the cytoplasmic region of APP, and APP phosphorylation play a central role in cellular responses to hyperosmotic stress).

Previously, we showed that the APP intracellular domain (AICD) fragment, which is generated by the serial cleavage of APP by α-secretase and γ-secretase, inhibits the nuclear-matrix accumulation of FE65 in osmotically stressed cells [7]. This observation seems to be in agreement with the present findings, as the PI2 domain of FE65 and APP phosphorylation play a central role in cellular responses to hyperosmotic stress.

Second, the N-terminal region of FE65, which includes the WW domain, plays an important role in the stabilization of p53 in osmotically stressed cells. Previously, we showed that the proline-rich region of p53 is required for the nuclear-matrix accumulation of this protein in osmotically stressed cells [8]. In general, the WW domain recognizes the proline-rich sequence [14]. However, p53 does not interact directly with FE65 [8] and the N-terminal region of FE65 was required for the prevention of the reduction in the viability of cells after long-term sorbitol treatment. Our previous and current results suggest that the N-terminal region of FE65 may function to prevent p53 degradation during osmotic stress by suppressing the proteasome degradation system [8].

Because the metabolic stabilization of p53 via this non-canonical pathway may be important for cellular stress responses [8], understanding the mechanisms that underlie FE65 trans-accumulation in the nuclear matrix may provide novel insights into how cells respond and survive to cellular stresses, which in turn will promote the understanding of the pathobiology of neurodegenerative diseases, including Alzheimer’s disease.

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References


Fig. 3. The PI2 domain was required for the nuclear matrix trans-accumulation of FE65. (A) Schematic structure of the FE65 proteins. EGFP-ΔPI1ΔPI2, EGFP-ΔPI1, EGFP-ΔPI2, and EGFP-PI2, which were tagged C-terminally with EGFP, are shown. The three protein-interaction domains and the numbers were described in Fig. 1. (B) Subnuclear distribution of the FE65 proteins. Cells expressing the various proteins were treated with (+) or without (−) sorbitol, subjected to subnuclear fractionation, and analyzed as described in Fig. 1B. (C) Recovery of FE65 proteins in the nuclear matrix. Proteins a, EGFP-ΔPI1ΔPI2; b, EGFP-ΔPI1; c, EGFP-ΔPI2; and d, EGFP-PI2) in the CL and NM fractions of panel b were quantified and their net recoveries (panel e) were calculated as described in Fig. 1. Data represent the mean ± S.D. (n = 3). ***P < 0.005, as assessed using a Student’s t-test against EGFP-full (in Fig. 1C, panel d).

