FE65, a neural adaptor protein, interacts with amyloid β-protein precursor (APP) and is known to regulate amyloid β generation from APP. FE65 also associates with nuclear proteins; however, its physiological function in the nucleus remains unclear. A fixed population of cytoplasmic FE65 is tethered to membranes by binding APP. This membrane-tethered FE65 is liberated from membranes by APP phosphorylation, which is facilitated by a stress-activated protein kinase in sorbitol-treated cells. Here we show that liberated FE65, which is distinct from “virgin” FE65 in the cytoplasm, translocates into the nucleus and accumulates in the nuclear matrix forming a patched structure. Targeting of FE65 into the nuclear matrix was suppressed by the APP intracellular domain fragment, which is generated by consecutive cleavages of APP. Thus, nuclear translocation of FE65 is under the regulation of APP. In the nucleus, FE65 induced γH2AX, which plays an important role in DNA repair as a cellular response by stress-damaged cells. These observations suggest that APP-regulated FE65 plays an important role in the early stress response of cells and that FE65 deregulated from APP induces apoptosis.

FE65 is a neural adaptor protein with several protein interaction domains, including one WW and two phosphotyrosine interaction/binding domains (1, 2). One FE65-interacting protein is amyloid β-protein precursor (APP), which is a causative factor in Alzheimer disease. It is well known that consecutive cleavages of APP result in extracellular secretion of a large amyloid α-peptide of APP (Aβ, the product of β- and γ-site cleavages) or the p3 fragment (the product of α- and γ-site cleavages) along with concomitant release of APP intracellular domain fragment (AICD) into the cytoplasm (3). Association of FE65 with APP regulates APP metabolism (4, 5) and is controlled by APP phosphorylation at Thr668, which is located 13 amino acids from the amino-terminal end of the FE65-binding GYENPTY motif (numbering for the APP695 isoform) (5). FE65 exhibits gene transactivation activity in the presence of AICD in the nucleus (6), although the relevancy of AICD in FE65-dependent gene transactivation still needs to be confirmed (7).

FE65 is largely located in cytoplasm, although moderate amounts of FE65 are tethered to membranes through binding with APP (8, 9). This membrane-tethered FE65 is liberated from the membrane by APP phosphorylation at Thr668 (9). Although several protein kinases have been implicated in this phosphorylation (10–13), a stress-activated protein kinase could phosphorylate APP at Thr668 and contribute to FE65 release from the membrane in cells under osmotic stress (9). Therefore, it has been hypothesized that FE65, along with APP, plays an important role in signal transduction during cellular stress responses. Here we investigated FE65 function in the cellular response of cells under hyperosmotic stress. FE65 translocation into the nuclear matrix of cells under osmotic stress was regulated by APP and its metabolic fragment, AICD. FE65 that was translocated into the nucleus under the regulation of APP induced γH2AX, which plays an important role in the DNA repair process. Therefore, APP-regulated FE65 contributes to the process of signal transduction in response to cellular stress.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Mouse monoclonal anti-α-tubulin (236-10501, Zymed Laboratories Inc./Invitrogen), anti-actin (C4, Chemicon/Millipore), anti-hemagglutinin (12CA5, Roche Applied Science), and anti-FLAG (M2, Sigma) antibodies and rabbit polyclonal anti-APP carboxyl-terminal APP/C (Sigma), anti-phospho-Thr668-APP (Immuno-Biological Laboratories, Co. Ltd.), anti-histone H1 (Santa Cruz Biotechnology, Inc.), and anti-EGFP (Clontech) antibodies were purchased from the indicated suppliers. Horseradish peroxidase-conjugated goat polyclonal anti-rabbit and anti-mouse IgGs were purchased from Amersham Biosciences. The rabbit polyclonal anti-Fe65 UT122 antibody used was described previously (9).

**Photobleaching Studies, Immunostaining of Cells, and Immunoblotting of Proteins**—Fluorescence photobleaching was achieved under a Carl Zeiss confocal laser scanning microscope with a photobleaching system (LSM510). Photobleaching procedures were performed according to the manufacturer’s pro-
tocol. Fluorescence quantification was performed using the imaging software ImageJ (National Institutes of Health).

The procedure for immunostaining of cells was described previously (9). In brief, mouse neuroblastoma Neuro2a (N2a) cells cultured on an 8-well chamber slide glass (Nalge Nunc International) were transfected with the indicated plasmids in Lipofectamine 2000 for 24 h, and the cells were fixed with 3.7% (w/v) formaldehyde in PBS for 10 min at room temperature (rt), permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min, and blocked with PBS containing 3% (w/v) bovine serum albumin. The cells were rinsed with PBS and incubated with the indicated antibodies overnight at 4 °C followed by incubation with goat anti-mouse IgG coupled with Alexa Fluor 546 secondary antibodies (Molecular Probe, Eugene, OR). After incubation with secondary antibody, cells were treated with 4',6-diamidino-2-phenylindole for 5 min followed by washing three times with PBS and observed under a confocal laser scanning microscope (LSM510, Carl Zeiss).

Immunoblotting of proteins has been described previously (9). Immunoreactive proteins were detected by ECL (GE Healthcare) and quantified with the VersaDoc imaging system (Bio-Rad).

**Culture, Gene Transfection, and Sorbitol Treatment of Cells**—N2a cells were cultured and transiently transfected with the indicated plasmids in Lipofectamine 2000 (Invitrogen) (9). To establish stable cell lines in which APP or FE65 expression was knocked down, N2a cells were transfected with pSUPERmAPP, pSUPERmFE65, or pSUPERcontrol vectors in Lipofectamine 2000 and screened by immunoblotting to examine endogenous expression of APP or FE65. Sorbitol treatment of cells was initiated by replacing the culture medium with medium containing 0.5 M sorbitol for the indicated times and completed by replacing the medium with medium lacking sorbitol.

**Plasmid Construction**—To prepare pEGFP-FE65, a cDNA encoding full-length human FE65 was amplified from pcDNA3.1NFLAG-FE65 (9) using the following primers: forward (f), 5'-gagccgccgtttgagagagagtttcaagagaatgctgcccttcctcaagtc-3' and reverse (r), 5'-ttccctcaaagccggg-3'. The amplified fragment was digested with KpnI/BamHI and cloned into the same cleavage sites of the pEGFP-N3 vector. pEGFP-AICDwt was generated by amplifying cDNA encoding AICD (from amino acids 653–695 of APP695) from pcDNA3APP695 (9) by PCR with the 5'-gagccgccgtttgagagagagtttcaagagaatgctgcccttcctcaagtc-3' (f) and 5'-ttccctcaaagccggg-3' (r) primers, digested with HindIII/XbaI, and cloned into the pcDNA3.1NFLAG-FE65 vector. The resulting fragment encoding mRFP was ligated into pECFP-Golgi (Clontech) in place of cDNA encoding enhanced cyan fluorescent protein using BamHI/NotI restriction sites. Then mRFP-FE65 was recloned into the pcDNA3.1 vector to obtain pcDNA3.1mRFP-FE65. To prepare pSUPERmAPP or pSUPERmAPP, two oligonucleotides (5'-gatcccgcggtaccgccaccatgtctgttcca-3' (f) and 5'-ttccctcaaagccggg-3' (r) for mouse FE65 and 5'-gatcccgcggtaccgccaccatgtctgttcca-3' (f) and 5'-aaagaaggccgttatccagcattctcttgaaatgctggataacggccttcttttta-3' (r) for mouse APP) were synthesized, annealed, and inserted into the pSUPERvector (OligoEngine) at the BglII/HindIII sites. pSUPER-control was prepared using the synthesized oligonucleotides 5'-gagccgccgtttgagagagagtttcaagagaatgctgcccttcctcaagtc-3' and 5'-ttccctcaaagccggg-3' (r) for FE65 or 5'-gagccgccgtttgagagagagtttcaagagaatgctgcccttcctcaagtc-3' and 5'-aaagaaggccgttatccagcattctcttgaaatgctggataacggccttcttttta-3' (r) and 5'-aaagaaggccgttatccagcattctcttgaaatgctggataacggccttcttttta-3' (f) and 5'-aaagaaggccgttatccagcattctcttgaaatgctggataacggccttcttttta-3' (r) for APP as described above. pEGFP-APP, pcDNA3NFLAG-APP, pcDNA3.1NFLAG-FE65 and pcDNA3.1NHA-FE65 were described in previous reports (5, 13, 14).

**Subcellular and Subnuclear Fractionation of Cultured Cells and Protein Analysis**—Subcellular fractionation was carried out as described previously (9). Subnuclear fractionation was also carried out as described previously (15) with minor modifications. Briefly cells were collected by centrifugation at 2500 × g for 10 min at 4 °C and suspended in CSK buffer (10 mM Pipes-NaOH (pH 6.8), 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 5 μg/ml chymostatin, and 5 μg/ml leupeptin) containing 100 mM NaCl and incubated for 3 min on ice. A 10% volume aliquot was lysed by sonication to prepare the crude cell lysate (CL). The CSK buffer suspension was centrifuged at 2500 × g for 10 min at 4 °C, and the supernatant was used as the membrane/lysosomal (MC) fraction. The pellet was resuspended in CSK buffer containing 250 mM ammonium sulfate and incubated on ice for 5 min. The suspension was then centrifuged at 10,000 × g for 2 min, and the supernatant was used as the nucleoplasm (NP) fraction. The pellet was washed with PBS, resuspended in CSK buffer containing 50 mM NaCl and DNase I (100 μg/ml), and incubated at rt for 20 min. After incubation, a 10% volume of 2.5 M ammonium sulfate was added, the solution was centrifuged, and the supernatant was collected as the DNA-binding protein fraction. The pellet was washed with PBS and then resuspended in CSK buffer containing 100 mM NaCl and RNase A (25 μg/ml). After incubation for 10 min at rt, the sample was centrifuged, and the supernatant was collected as the RNA-binding protein fraction. The washed pellet was resuspended in Tris/SDS solution (10 mM Tris-HCl (pH 7.4), 1% (w/v) SDS, 5 μg/ml chymo-
DNA was extracted using phenol-chloroform, precipitated by Student's antibody. FE65 distribution in cells with (absence of sorbitol (withdrawal; samples were cultured in the presence of sorbitol for 45 min and then further cultured for 1 or 3 h in the presence of sorbitol). Some samples were cultured in the presence of sorbitol for 45 min and then further cultured for 1 or 3 h in the absence of sorbitol (withdrawal; lanes 5, 6, 11, 12, 17, and 18). Cells were fractionated into membrane (lanes 1–6), cytosol (lanes 7–12), and nuclear (lanes 13–18) fractions. Equal amounts of protein (20 μg) were immunoblotted with antibodies against FE65, APP, APP phosphorylated at Thr668 (pAPP), α-tubulin, or histone H1. Arrows, proteins specifically detected by the antibodies. B, subnuclear distribution of FE65 in sorbitol-treated cells. N2a cells (5 × 10⁵) expressing NFLAG-cp2 and NFLAG-hnRNP L were treated with (0.5M sorbitol) or without (−) sorbitol for 45 min and lysed. The CL was fractionated into MC, NP, DNA-bp, RNA-bp, and NM fractions. Equal volumes of the obtained fraction (one-sixth volume for MC, NP, DNA-bp, RNA-bp, and NM) except for CL (one-thirtieth volume) were analyzed by immunoblotting with the antibodies described in A and anti-FLAG M2 antibody. FE65 distribution in cells with (+) or without (−) sorbitol treatment is indicated as percent recovery with FE65 in the CL fraction normalized to 100%. Data represent mean ± S.D. for n = 3 experiments. *, p < 0.02 by Student’s t test. Error bars indicate S.D. with ethanol, and then dissolved in double distilled water containing RNase A (20 μg/ml) and incubated for 30 min at rt. DNA was then subjected to agarose (1%) gel electrophoresis and stained with EtBr.

**Comet Assay**—Cells were suspended in 1% (w/v) low melting agarose (Sigma A6560) in PBS and dropped onto a glass plate coated with 1% (w/v) agarose HS (Nippon gene). The plate was covered with a coverslip for 5 min at rt. The coverslip was removed, and the glass plate was soaked in a lysis buffer (2.5 mM NaCl, 0.1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 10% DMSO, and 1% Triton X-100) for 1.5 h at 4 °C, washed with 10 mM Tris-HCl (pH 8.0), and left in neutral electrophoresis buffer (300 mM NaOAc, 100 mM Tris-HCl (pH 8.3), and 1% DMSO) for 45 min at 4 °C. The samples were then subjected to electrophoresis for 40 min at 25 V at 4 °C and washed three times with 0.4 M Tris-HCl (pH 7.4). The glass plate was then soaked in 70% ethanol for a few minutes to dehydrate, completely dried, and stained with 4',6-diamidino-2-phenylindole. Samples were covered with a coverslip and observed with a confocal microscope (LSM510, Carl Zeiss).

**Flow Cytometry**—Cells were treated with 70% ethanol and stored at −20 °C until analysis. The stored cells were precipitated by centrifugation and washed with PBS twice, incubated with stain solution (PBS containing 50 μg/ml propidium iodide, 100 μg/ml boiled RNase A, and 0.05% Triton X-100 in PBS) for 30 min at rt, and analyzed with FACSort (BD Biosciences).

**RESULTS**

**Nuclear Accumulation of FE65 in Cells Treated with Sorbitol**—A previous report indicates that membrane-tethered FE65 is released into an FE65-rich cytoplasm (9). To assess the fate of FE65 released from APP into the cytoplasm of cells under osmotic stress, we examined subcellular fractions and identified cellular localization of FE65. In N2a cells, FE65 largely localized in the cytoplasm as expected, whereas a fixed amount of FE65 was recovered from the membrane fraction, and small amounts of FE65 were detected in the nucleus (Fig. 1A, lanes 1, 7, and 13). When N2a cells were treated with sorbitol, the level of APP phosphorylation at...
Thr668 increased, the amount of membrane-tethered FE65 decreased, and the level of FE65 in the nucleus increased in a time-dependent manner (Fig. 1A, lanes 1–4 and 13–16). Sorbitol removal induced dephosphorylation of APP, disappearance of nuclear FE65, and restoration of membrane-tethered FE65 (Fig. 1A, lanes 5, 6, 17, and 18). Nuclear FE65 may be exported or degraded in the nucleus when hyperosmotic conditions are abolished. This enhanced nuclear localization of FE65 in response to sorbitol was also observed in COS7, SH-SY5Y, HEK293, and mouse central nervous system catecholaminergic CAD cells as well as in response to other osmolytes such as mannitol or NaCl and genotoxic stresses such as H2O2 (data not shown).

To determine which nuclear region showed FE65 accumulation after sorbitol treatment (Fig. 1B), N2a cells treated with (+) or without (−) sorbitol were lysed, and the CLs were fractionated into MC and nuclear fractions. The nuclear fraction was further subfractionated into NP, DNA-binding protein (DNA-bp), RNA-binding protein (RNA-bp), and NM fractions. Intranuclear FE65 distribution in cells treated with (+) or without (−) sorbitol was analyzed by immunoblotting (Fig. 1B, upper panels), and quantitative recovery was determined as the percent yield (Fig. 1B, lower graph).

Regardless of sorbitol treatment, FE65 was largely recovered in the MC fraction with a modest NP fraction also recovered. Approximately 20% of FE65 was recovered in the NM fraction in sorbitol-treated cells. Negligible FE65 was detected in the DNA-bp and RNA-bp fractions. In contrast, the transcriptional regulator CP2/LSF/LBP1 (designed cp2) was detected in the DNA-bp fraction, and ~50% of the pre-mRNA-binding protein heterogeneous nuclear ribonucleoprotein L (designed hnRNP L) was recovered in the RNA-bp fraction (Fig. 1B, upper panels). These observations, together with previous reports (9), suggest that some of the FE65 population, perhaps those that are membrane-tethered, translocated into the nucleus, particularly into the nuclear matrix, in response to sorbitol exposure.

Cellular Localization of FE65 in Living Cells with or without Sorbitol Treatment—Although we hypothesize that membrane-tethered FE65 translocated into the nuclei (Fig. 1A), it was difficult to distinguish membrane-released from cytoplasm-derived FE65. Therefore, we observed real-time movement of EGFP-FE65 in living cells with or without sorbitol...
treatment. EGFP-FE65 was located diffusely throughout the cell, although a slight accumulation was observed at the perinuclear membrane region that largely co-localized with Golgi (Fig. 2A). When EGFP-FE65 was co-expressed with APP695, EGFP-FE65 was significantly localized around the perinuclear region with a few diffuse distributions in the cytoplasm and nucleus (Fig. 2B). The majority of APP695 localized to the perinuclear region primarily at the Golgi (Fig. 2C), suggesting that EGFP-FE65 could be tethered at the membrane via binding to APP. Co-localization of APP and FE65 at the perinuclear region was confirmed by immunostaining of fixed cells (data not shown).

To examine FE65 translocation in living cells, we performed fluorescence photobleaching. When the perinuclear region was subjected one time to photobleaching (the bleached region is indicated within a red circle in Fig. 2D), the majority of EGFP-FE65 at the perinuclear region was bleached out and then reappeared after bleaching (Fig. 2D). However, continuous bleaching to EGFP-FE65 at the perinuclear region abolished fluorescence of EGFP-FE65 throughout the cell (Fig. 2E). This is due to a continuous photobleaching at the perinuclear region rather than the photobleaching throughout the cell (data not shown).

When cells expressing EGFP-FE65 with APP695 were treated with 0.5 M sorbitol, EGFP-FE65 localized as "patches" in the nuclei (Fig. 3B). Herein the patches are assigned to the shape of the region where EGFP-FE65 accumulated in nuclei and do not indicate a specific subnuclear structure. These patches were not observed in nuclei prior to sorbitol treatment (Fig. 3A). The fluorescence of EGFP-FE65 in the nuclei became stronger with increasing time of sorbitol treatment (Fig. 3C). These results suggest that FE65 enters the nuclei and forms patched structures in cells following sorbitol treatment.

To confirm the translocation of FE65 into the nucleus from the perinuclear membrane region, we photobleached the sorbitol-treated cells. When EGFP-FE65 patches in the nucleus were bleached one time following 20 min of sorbitol treatment, the fluorescence disappeared and then reappeared in a time-dependent manner, although the level was not restored completely (Fig. 4, A and D). When EGFP-FE65 at the perinuclear region was bleached one time following 20 min of sorbitol treatment, the fluorescence disappeared and then reappeared at the bleached region (Fig. 4, B and E). The nuclear patches of EGFP-FE65 were largely unaf-
FE65 Nuclear Translocation Regulated by APP

In sorbitol-treated cells, EGFP-FE65 fluorescence at the perinuclear region was bleached at the perinuclear region. In contrast, when cells were subjected to continuous bleaching at the perinuclear region after 20 min of sorbitol treatment, the fluorescence of EGFP-FE65 in both the cytoplasm and nuclear patches disappeared (Fig. 4, C and F). These results suggest that in cells under osmotic stress APP-tethered FE65 in the perinuclear region translocated into the nucleus, and a fixed ratio of FE65 in the cytoplasmic pool became newly tethered at the perinuclear membrane region. This result is consistent with our previous observations that APP-tethered FE65 is released from membranes by APP phosphorylation (9).

We also observed nuclear patch formation by EGFP-FE65 in cells without APP overexpression (Fig. 5A, top panels). Photobleaching experiments of EGFP-FE65 in such cells gave results...
similar to those in cells with APP overexpression (supplemental Fig. S1; compare with Fig. 4). The reason for this was thought to arise because of the presence of a moderate level of endogenous APP. To confirm the role of APP in this process, we used small interfering RNA to generate the APP knockdown cell line C5, which has APP levels that are ~20% of those in parental N2a and control A3 cell lines (supplemental Fig. S2). When A3 or N2a cells expressing EGFP-FE65 were treated with sorbitol, EGFP-FE65 formed nuclear patches in a time-dependent manner (Fig. 5A, top and bottom panels). In contrast, almost no nuclear patch formation of EGFP-FE65 was observed in sorbitol-treated C5 cells expressing EGFP-FE65 (Fig. 5A, middle panels). In A3 cells, ~20% of the total amount of endogenous FE65 accumulated in the nuclear matrix fraction after sorbitol treatment.
treatment, whereas in C5 cells a reduced level of endogenous FE65 accumulated in the nuclear matrix fraction after sorbitol treatment (Fig. 5B).

In C5 cells, overexpressed EGFP-FE65 did not form nuclear patches (Fig. 5A), although a moderate amount of endogenous FE65 was detected in the nuclear matrix even when endogenous levels of FE65 were very low (Fig. 5B). These results suggest that a fixed ratio of APP to FE65 may be needed for sorbitol-induced nuclear matrix targeting of FE65. Therefore, we next examined the efficiency of FE65 translocating into the nuclear matrix of cells differentially expressing APP. Cells expressing a fixed amount of NHA-FE65 (FE65 with an amino-terminal hemagglutinin tag) were transfected with various amounts of pcDNA3NFLAG-APP and treated with or without sorbitol, and proteins were fractionated into MC and nuclear (N) fractions. Proteins were immunoblotted with antibodies to the APP cytoplasmic domain, FE65, or histone H1. The band intensities of NHA-FE65 were quantified, and protein recovery in the N is indicated relative to CL.

AICD Suppresses FE65 Localization to the Patched Nuclear Matrix—FE65 interacts not only with APP but also with AICD, which is believed to function in nuclei (6, 17, 18). Indeed AICD was detected in the nuclear fraction in vivo (9). These observations led us to hypothesize that nuclear FE65 associates with AICD to enhance some functions in which case the proteins may co-localize in the nuclei. Therefore, we examined localization of AICD and FE65 in sorbitol-treated cells. We used EGFP fusions with AICD wild type (AICDwt) or with an AICD mutant (AICDmt) in which Tyr<sup>682</sup> and Tyr<sup>687</sup> of APP<sup>695</sup> were substituted with Ala rendering the mutant unable to interact with FE65 (19). Furthermore AICD-EGFP is metabolically more stable than AICD in the cell (data not shown).

N2a cells co-expressing NFLAG-FE65 with AICDwt-EGFP or AICDmt-EGFP were treated with (+ sorbitol) or without (control) sorbitol, and the nuclear localization of FE65 was examined (Fig. 6A). NFLAG-FE65 in the presence of AICDwt-EGFP showed a pan-cellular localization in control cells (Fig. 6A, panels a–d) similar to the localization in cells expressing EGFP-FE65 alone (Fig. 2A). Unexpectedly in sorbitol-treated cells, the majority of NFLAG-FE65 in the presence of AICDwt-EGFP was detected throughout the cell with a few NFLAG-FE65 proteins demonstrating a weak, patchy co-localization with AICDwt-EGFP in the nucleus (Fig. 6A, panels e–h). This result was quite different from the results from cells with EGFP-FE65 alone. In contrast, NFLAG-FE65 in the presence of AICDmt-EGFP showed a pan-cellular localization in control cells.

**FIGURE 6.** AICD negatively regulates FE65 localization to nuclear patches and nuclear matrix in sorbitol-treated cells. A, AICD inhibits FE65 localization to nuclear patches. N2a cells expressing NFLAG-FE65 and either AICDwt-EGFP (panels a–h) or AICDmt-EGFP (panels i–p) were treated with (+ sorbitol) or without (control) sorbitol for 45 min. The cells were fixed, and EGFP fluorescence (panels a, e, i, and m) was observed together with anti-FLAG (panels b, f, j, and n) and 4′,6-diamidino-2-phenylindole (DAPI) (panels c, g, k, and o) staining. Scale bar, 5 μm. B, AICD inhibits nuclear matrix distribution of FE65. N2a cells (5 × 10<sup>5</sup>) expressing NFLAG-FE65 and EGFP, AICDwt-EGFP, or AICDmt-EGFP were treated with (+ sorbitol) or without (control) sorbitol for 45 min and subjected to subcellular fractionation. CL (one-thirtieth volume of obtained lysate) and NM (one-sixth volume of obtained fraction) fractions were immunoblotted with antibodies to FLAG, EGFP, or actin. The band intensities of NFLAG-FE65 were quantified, and protein recovery in the NM is indicated relative to CL.

* p < 0.05 for n = 3 by Student’s t test. C, localization of endogenous AICD to nuclei of sorbitol-treated cells. N2a cells (5 × 10<sup>5</sup>) treated with (+) or without (−) sorbitol were subjected to subcellular fractionation. The cells were lysed to prepare CL, and proteins were fractionated into MC and nuclear (N) fractions. Proteins were immunoblotted with antibodies to the APP cytoplasmic domain, FE65, or histone H1. The second panel for AICD shows a longer exposure of part of the top panel. The protein band intensities of AICD were quantified, and the nuclear AICD levels are indicated at the right relative to cells without sorbitol treatment (−). ***, p < 0.005 by Student’s t test. Data represent mean ± S.D. for n = 3 experiments. Error bars indicate S.D. APP CTFα, carboxyl-terminal fragment of APP cleaved at the α-site.
FE65 Nuclear Translocation Regulated by APP

(Fig. 6A, panels i–l) and a patched nuclear localization in sorbitol-treated cells (Fig. 6A, panels m–p) similar to the results with EGFP-FE65 alone (Fig. 4 and supplemental Fig. S1).

These microscopic observations were confirmed by nuclear fractionation (Fig. 6B, upper panel). The yield of NFLAG-FE65 in the nuclear matrix fraction was extremely low in the presence of AICDwt-EGFP in sorbitol-treated cells (Fig. 6B, lane 11) compared with cells co-expressing EGFP (Fig. 6B, lane 10) or AICDmt-EGFP (Fig. 6B, lane 12). The amount of NFLAG-FE65 in the nuclear matrix of cells expressing AICDwt-EGFP was ~50% of that in cells expressing EGFP alone or AICDmt-EGFP (Fig. 6B, lower graph). AICD appeared to suppress nuclear matrix localization of FE65. Although almost identical amounts of AICDwt-EGFP and AICDmt-EGFP were expressed in the cells (Fig. 6B, lanes 2, 3, 5, and 6), less AICDmt-EGFP was recovered from the NM (lanes 9 and 12) compared with AICDwt-EGFP (lanes 8 and 11). The nuclear matrix localization of AICD, which also was observed in our microscopy results, may be suppressed by the amino acid substitutions in the mutant (Fig. 6A, compare panels e and m).

The relationship between the nuclear amounts of endogenous FE65 and AICD was also examined (Fig. 6C). In sorbitol-treated N2a cells (+), the endogenous AICD level in the nucleus was reduced up to ~50% compared with untreated cells (−) (Fig. 6C, left panel, second row). In contrast, the accumulation of FE65 was increased in the nucleus of sorbitol-treated cells (Fig. 6C, left panel, third row). We could not perform subnuclear fractionation of endogenous AICD because very small amounts of AICD were present, and the low molecular weight of AICD resulted in its diffusion from the nucleus during subnuclear preparation. Thus, it is not clear whether the amount of endogenous AICD in the nuclear matrix fraction changed in response to sorbitol treatment. However, together with studies using metabolically stable AICD-EGFP, these results suggest that AICD regulates the intranuclear localization of FE65 and, in particular, suppresses the translocation of FE65 into the nuclear matrix of sorbitol-treated cells.

FE65 Nuclear Accumulation Occurs Prior to γH2AX Induction during Sorbitol-induced DNA Damage—Hyperosmotic stress is a cell stress that induces various cellular responses, including DNA damage (20). Other genotoxic reagents induce nuclear accumulation of FE65 (21). These reports suggest some role for FE65 during DNA damage in sorbitol-treated cells.

To test this, we examined the time course of FE65 appearance in the nucleus and compared it with the DNA damage response (Fig. 7). We examined the time course of DNA fragmentation in sorbitol-treated cells using the comet assay. After 45 min of sorbitol treatment, we did not observe a remarkable increase in the comet tail (Fig. 7A, + sorbitol) compared with control (Fig. 7A, control). However, when sorbitol was removed from the medium and cells were cultured for an additional 45 min, comet tails were observed in many cells (Fig. 7A, withdrawal). This was confirmed by examining DNA fragmentation (Fig. 7B), which was not increased in sorbitol-treated cells (+ s 45′) but markedly enhanced in cells further cultured for 45 min in the absence of sorbitol (withdrawal 45′). DNA fragmentation in cells had decreased by 6 h after sorbitol withdrawal.
It is known that DNA damage induces the phosphorylation of histone H2AX (phosphorylated H2AX is designated as γH2AX) (22). In sorbitol-treated cells, γH2AX was induced and reached a maximal level in cells that were further cultured for 45 min to 3 h in the absence of sorbitol after 45-min treatment (Fig. 7C, withdrawal 45' and 3h). γH2AX decreased at 6 h and returned to basal levels by 24 h after withdrawal of sorbitol (Fig. 7C and data not shown). This time course of H2AX phosphorylation and dephosphorylation was consistent with the time course of DNA fragmentation and restoration (Fig. 7, compare B and C).

We also found that nuclear accumulation of FE65 occurred prior to γH2AX induction in sorbitol-treated cells (Fig. 7D). Small amounts of FE65 and γH2AX were detected in the nuclei of cells (control). When the cells were treated with sorbitol (+sorbitol) for 45 min, FE65 in the nucleus reached a maximal level, whereas the appearance of γH2AX required an additional 45 min in culture (Fig. 7D, withdrawal). These observations suggest that FE65 functions prior to γH2AX induction in response to cellular stress.

**FE65 Regulates γH2AX Induction in Sorbitol-Treated Cells**—If nuclear accumulation of FE65 plays an important role in the response of cells to DNA-damaging stress, the reduction of FE65 levels may impair the DNA damage response such as the appearance of γH2AX in sorbitol-treated cells. To examine this possibility, we generated FE65 knockdown cell lines, B2 and E5, in which the expression of endogenous FE65 was reduced to ~20% (B2) and 40% (E5) of the parental N2a cell line or the control small interfering RNA cell line, F7 (supplemental Fig. S3).

To analyze the role of FE65 in the induction of γH2AX, B2 and F7 cells were treated with sorbitol for 45 min and further cultured for 15–135 min in the absence of sorbitol (withdrawal). The appearance of γH2AX was then examined by immunoblotting (Fig. 8). In control F7 cells, which express FE65 at levels similar to those in parental N2a cells, γH2AX began to increase by 15 min after sorbitol withdrawal (Fig. 8A, lane 12), reached a maximum at 45–60 min after withdrawal (Fig. 8A, lanes 14 and 15), and then decreased at 120–135 min after withdrawal (Fig. 8A, lanes 17 and 18). This time course was similar to that observed in parental N2a cells (data not shown). In B2 cells, which express FE65 at reduced levels, the appearance of γH2AX after sorbitol treatment followed a time course similar to that observed in control cells (Fig. 8A, lanes 2–9, upper panel). However, the level of γH2AX in B2 cells was significantly lowered at 45 and 60 min after sorbitol withdrawal (Fig. 8A, lower panel, asterisks) than that in F7 cells. Lower levels of γH2AX induction were also observed in the other FE65 small interfering RNA cell line, E5 (supplemental Fig. S3).

The suppression of γH2AX induction in B2 cells was rescued by an overexpression of NFLAG-FE65 (Fig. 8B, upper panel), and the γH2AX level increased by ~1.5-fold at 45 min after sorbitol withdrawal compared with B2 cells not overexpressing NFLAG-FE65 (control) (Fig. 8B, lower graph). Unexpectedly we observed a long lasting increase of γH2AX induction by FE65 overexpression compared with control cells (compare the protein bands at 135 min after withdrawal of sorbitol). This suggests that the well regulated translocation of FE65 into the nucleus may be necessary for the accurate induction of γH2AX during DNA repair.

**FE65 Overexpression Rescues Osmotic Stress-induced Apoptosis and Facilitates Apoptosis in Unstressed Cells**—We noted that the long lasting increase of γH2AX because of the overexpression of FE65 may impair the damage-induced DNA repair system and cell apoptosis. Therefore, we investigated the levels of sorbitol-induced apoptosis in N2a, B2, and F7 cells (Fig. 9). Cells remaining at the sub-G1 phase of the cell cycle, as determined by cytometric analysis, were defined as apoptotic (23). Untreated N2a, B2, and F7 cells had low percentages (~5%) of apoptotic cells (Fig. 9A, left panels, and quantified in Fig. 9C, panel a) with no significant differences between the cell lines. When the cells were treated with sorbitol for 24 h, each of the cell lines had significantly increased populations of apoptotic cells (Fig. 9A, right panels, and quantified in Fig. 9C, panel a, sorbitol columns). B2 cells had a significantly higher apoptotic level than the N2a and F7 cell lines (Fig. 9, A and C, panel a).

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**FIGURE 8. Induction of γH2AX by FE65 in sorbitol-treated cells.** A, suppression of γH2AX induction in cells expressing reduced levels of FE65. Upper panel, B2 cells, which have reduced expression of FE65, and control F7 cells were treated with (lanes 2–9 and 11–18) or without (control; lanes 1 and 10) sorbitol for 45 min and were further cultured without sorbitol (withdrawal) for the indicated times. The cells were lysed and immunoblotted for γH2AX, or actin. Lower panel, γH2AX induction was quantified in B2 (open circles) and F7 (closed circles) cells relative to actin with control values set to 1.0. Data represent mean ± S.D. for n = 3 experiments. *, p < 0.05 by Student’s t test. B, restoration of γH2AX induction in B2 cells by FE65 overexpression. Left panel, B2 cells with (NFLAG-FE65; lanes 7–12) or without (control; lanes 1–6) overexpression of NFLAG-FE65 were treated with (lanes 2–6 and 8–12) or without (control; lanes 1 and 7) sorbitol for 45 min. The cells were further incubated without sorbitol for the indicated times (withdrawal; lanes 2–6 and 8–12). B2 cells were lysed and immunoblotted with antibodies to γH2AX, or actin. Right panel, the protein band intensities of γH2AX at 45 min after sorbitol withdrawal in cells with (lane 11) or without (lane 5) FE65 overexpression were quantified and normalized to actin with control values set to 1.0. Data represent mean ± S.D. for n = 3 experiments. *, p < 0.05 by Student’s t test. Error bars indicate S.D. C, control.
When B2 cells expressing EGFP were treated with sorbitol for 24 h, they showed an increase in apoptosis similar to that observed without plasmid transfection (Fig. 9B, left panel, and quantified in Fig. 9C, panel b), whereas sorbitol-treated B2 cells expressing EGFP-FE65 had a decreased number of apoptotic cells (Fig. 9B, right panel, and quantified in Fig. 9C, panel b). We confirmed that overexpression of EGFP-FE65 in B2 cells also increased the γH2AX level as did NFLAG-FE65 shown in Fig. 8B (data not shown). The results indicate that cells with a low level of FE65 were vulnerable to stress, whereas overexpression of FE65 rescued cells from apoptosis. These observations contradicted the hypothesis that FE65 overexpression induces a long lasting increase of γH2AX that results in increased apoptosis of stressed cells (Fig. 8B). However, we also found that overexpression of FE65 induced increased apoptotic cell death when cells were not subjected to sorbitol treatment (Fig. 9D, panels a–c, and quantified in Fig. 9C, panel b). Our current data suggest that FE65 plays an important role in the prevention of apoptosis when cells are subjected to hyperosmotic stress, whereas excess FE65 facilitates apoptosis when cells are not subjected to stresses.

**DISCUSSION**

The neuronal adaptor protein FE65 associates with membrane proteins, including APP and nuclear proteins (6, 18, 24–26). Nuclear localization of FE65 has also been reported (17), and several studies have suggested that one potential function for FE65 in the nucleus may be in gene transactivation (6, 17, 18). FE65 was recently proposed to play a role in pre-mRNA splicing (27) and DNA damage repair (21). However, the precise function of FE65 in the nucleus and the mechanisms regulating FE65 translocation into the nucleus are still unclear.

Here we found that some population, very likely membrane-tethered, of cellular FE65 translocated into the nucleus following hyperosmotic stress of the cell. FE65 accumulates as a patched structure in the nuclear matrix, suggesting that FE65 mediates cellular responses to hyperosmotic stress. Cytoplasmic FE65 proteins are largely diffusible; however, moderate numbers of FE65 are tethered by membranes through binding to membrane proteins such as APP (Fig. 10, 1). There is also a small amount of nuclear FE65 in unstimulated cells (Fig. 10, 2). These results suggest that the nuclear localization of FE65 is tightly regulated.

When cells are responding to osmotic stress (Fig. 10, 3), membrane-tethered FE65 translocates into the nucleus by releasing from phosphorylated APP (Fig. 10, 4) as demonstrated by evidence that photobleaching of membrane-tethered FE65 at the perinuclear region induced the disappearance of

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**Figure 9.** Effect of FE65 on apoptosis in sorbitol-treated cells. A, role of FE65 in apoptosis suppression in sorbitol-treated cells. N2a, FE65 knockdown B2, and control F7 cells were treated with (right) or without (left) sorbitol for 24 h and then subjected to flow cytometric cell cycle analyses. Apoptotic cell populations were confirmed by evidence that photobleaching of membrane-tethered FE65 translocates into the nucleus by releasing from phosphorylated APP (Fig. 10, 4) as demonstrated by evidence that photobleaching of membrane-tethered FE65 at the perinuclear region induced the disappearance of
nuclear patched FE65. In cells with reduced levels of APP, nuclear targeting and patch formation of FE65 were rare events even when the cells were subjected to osmotic stress. Thus, it is reasonable to assume that nuclear translocation of FE65 in cells under osmotic stress is regulated by APP located at perinuclear membranes. Along with this, it is also reasonable that a moderate level of APP expression is required to regulate nuclear translocation of FE65 and for the cell to respond to osmotic stress. Too low or too high an amount of APP expression may impair the regulation of FE65 translocation into the nucleus. However, how APP modifies FE65 through their interaction remains unknown. We found that AICD, a metabolic cytoplasmic fragment derived from APP, suppressed the nuclear matrix accumulation of FE65 (Fig. 10). Overexpression of APP also constitutively generated excess AICD, which may disturb FE65 translocation. Therefore, the cellular level of APP is important for precise regulation of FE65 nuclear translocation.

We focused our research of FE65 on the DNA damage response and found that hyperosmotic stress-induced nuclear matrix accumulation of FE65 occurred prior to γH2AX induction. γH2AX is an essential factor in the repair of damaged DNA and appears early in most cellular responses to DNA damage (22). Although the detailed molecular mechanism remains unclear, our results suggest that γH2AX might be induced by nuclear localization of FE65. It is known that H2AX is phosphorylated by ataxia telangiectasia-mutated kinase (28). Therefore, nuclear translocation of FE65 may regulate ataxia telangiectasia-mutated kinase activity, although the possibility is a future issue to be revealed.

A larger percentage of cells with reduced levels of FE65 underwent apoptosis in response to osmotic stress, and unexpectedly, overexpression of FE65 increased the number of apoptotic unstrressed cells. These observations suggest that the well-regulated nuclear translocation of FE65 can induce precise responses to DNA damage, and in contrast, excess FE65 facilitates DNA damage in unstrressed cells. Taken together, these results indicate that the regulated nuclear translocation of FE65 plays an important role in the early stages of stress responses by cells. An attractive hypothesis is that FE65 transmits a stress signal to the nucleus and induces cellular responses to stress, such as induction of γH2AX in response to DNA fragmentation (Fig. 10).

The JNK-dependent protein phosphorylation cascade is a major cell signaling pathway, and it is worthwhile to note that activated JNK can phosphorylate APP (13, 29). APP phosphorylation liberates membrane-tethered FE65 and allows FE65 to translocate into the nucleus and induce γH2AX. Further studies are needed to reveal the mechanisms by which FE65 induces γH2AX in the nuclear matrix of cells in response to stress and the difference between FE65 associated with APP and “virgin” cytoplasmic FE65. One previous report indicates that FE65 suppresses the induction of γH2AX (21). However, this may not be contradictory to our results because well-organized nuclear translocation of FE65 is likely to be important in the regulation of the nuclear γH2AX level. In any case, these results indicate that APP-tethered FE65 plays an important role in signal transduction during cellular stress rather than its previously postulated role in gene transactivation (6).

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