Essential Roles for Fe65, Alzheimer Amyloid Precursor-binding Protein, in the Cellular Response to DNA Damage

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Fe65 interacts with the cytosolic domain of the Alzheimer amyloid precursor protein (APP). The functions of the Fe65 are still unknown. To address this point we generated Fe65 knockout (KO) mice. These mice do not show any obvious phenotype; however, when fibroblasts (mouse embryonic fibroblasts), isolated from Fe65 KO embryos, were exposed to low doses of DNA damaging agents, such as etoposide or H2O2, an increased sensitivity to genotoxic stress, compared with wild type animals, clearly emerged. Accordingly, brain extracts from Fe65 KO mice, exposed to non-lethal doses of ionizing radiations, showed high levels of γ-H2AX and p53, thus demonstrating a higher sensitivity to X-rays than wild type mice. Nuclear Fe65 is necessary to rescue the observed phenotype, and few minutes after the exposure of MEFs to DNA damaging agents, Fe65 undergoes phosphorylation in the nucleus. With a similar timing, the proteolytic processing of APP is rapidly affected by the genotoxic stress: in fact, the cleavage of the APP COOH-terminal fragments by γ-secretase is induced soon after the exposure of cells to etoposide, in a Fe65-dependent manner. These results demonstrate that Fe65 plays an essential role in the response of the cells to DNA damage.

Fe65, together with Fe65L1 and Fe65L2, belongs to a small protein family having the characteristics of adaptor proteins, as they possess three protein-protein interaction domains, one WW and two phosphotyrosine binding domains (1). All three proteins interact with the cytosolic domain of the β-amyloid precursor protein APP4 and with the cognate proteins APLP1 and APLP2, through their COOH-terminal phosphotyrosine binding domain (2–4). Fe65 also interacts, through its central phosphotyrosine binding domain with the low density lipoprotein-receptor-related protein LRP and with the ApoE receptor and experimental evidence indicates that a trimeric complex exists including LRP, Fe65, and APP (5–7). The same phosphotyrosine binding domain is also responsible for the interaction of Fe65 with the histone acetyl transferase Tip60 (8) and with the transcription factor CP2/LSF/LBP1 (9). The WW is involved in the interaction with Mena, the mammalian orthologue of the enabled protein of Drosophila (10), with the tyrosine kinase Abl (11) and with chromatin remodeling factor SET (12).

The function of this complex network of protein-protein interactions is still not understood. The involvement of Mena and Abl and the possible competition of Fe65 with Dab-1 for the interaction with APP support the results indicating the role of Fe65 in cytoskeleton remodeling and cell migration (13, 14). On the other hand, the interaction of Fe65 with several proteins whose main location is the nucleus has suggested that Fe65 could also have nuclear functions. This possibility is supported by the observations that overexpression of Fe65 results in its accumulation in the nucleus and APP functions as an extranuclear anchor that prevents Fe65 nuclear translocation (8, 15–18). Further experiments demonstrated that Fe65 activates the transcription of reporter genes and is associated in vitro with chromatin complexes (12, 19–21).

Relevant information about the possible functions of Fe65 comes from the analysis of knock-out animals. In Caenorhabditis elegans a single gene encodes a proteins very similar to the mammalian Fe65s. This orthologue, named feh-1, interacts with the nematode orthologue of APP (22). Heterozygous worms for the feh-1 gene deletion show a pharyngeal phenotype represented by a higher rate of pumping compared with the wild type animals. The ablation of both the feh-1 alleles results in the block of the worm development at the embryonic stage; a number of embryos escape this block and the development proceeds to the L1 larval stage (22). In the mouse, the double knock-out of Fe65 and Fe65L1 genes shows significant defects in the development of the central nervous system (23) that resemble those observed in the APP;APLP1;APLP2 gene knock-out mice (24).

Understanding the functions of Fe65 proteins is obviously linked to the relevance of APP and its proteolytic processing to Alzheimer disease (AD). There is in fact a convincing and large collection of data that supports the crucial role played by the β-amyloid peptides, derived from the processing of APP, in the
pathogenesis of the disease (25). However, the functions of APP and the mechanisms governing its amyloidogenic processing are not fully understood.

Herein we report results demonstrating that the ablation of the Fe65 gene in the mouse is associated with an increased sensitivity to DNA damaging agents and suggest that Fe65 is involved in the molecular machinery of the cellular response to genotoxic stress.

MATERIALS AND METHODS

Fe65 Knock-out Mice, Knock-out Mice Embryonic Fibroblast Generation—Fe65 KO mice were obtained as described in the supplemental material (part 1). For generation of mouse embryo fibroblast lines, E13.5 embryo bodies devoid of heads and liver were trypsinized and plated in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% Newborn Calf Serum (HyClone), 1% penicillin and streptomycin, 1% minimal essential medium-non-essential amino acids (Invitrogen), 1.6 mm 2-mercaptoethanol. Immortalized lines were obtained following spontaneous recovery of the primary cultures from proliferation crisis. These lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Chemicals and Treatments—Etoposide (VP-16, Calbiochem, 100 mm stock in MeSO) was used 15 or 40 μM. H2O2 was diluted from a 30% w/w aqueous solution (Sigma) immediately before cell treatment in complete medium. Chronic treatments with 2 μM γ-secretase inhibitor IX (Calbiochem) were performed by repeated supplementation of the compound, every 4 h, over 24 h.

To induce genotoxic stress in mice, these were exposed to 0.5 or 1 gray doses of 6-MV x-ray from a linear accelerator (Primus, Siemens).

Generation of the Recombinant Constructs and Transfections—pRC-CMV-NES-myc and pRC-CMV-mutNES-myc vectors were obtained by cloning in the pRC-CMV (Invitrogen) the sequence of NES-myc and mutNES-myc using NotI/ApaI restriction sites. NES-myc and mut NES-myc ds DNA were obtained by annealing NES-myc and mutNES-myc using NotI/ApaI restriction sites. NES-myc and mut NES-myc ds DNA were obtained by annealing synthetic oligonucleotides (see supplemental material (part 2)). The Fl65-NES and Fl65-NESm constructs were obtained by cloning into the pRC-CMV-NES-myc and pRC-CMV-mutNES-myc vectors, respectively, the Fe65 cDNA amplified by PCR with the primers reported in supplemental material (part 2) and digested with appropriate restriction enzymes. MEFs were transfected at 1 × 105 cells/well in 6-well plates and with 4 μg each of cDNA and 10 μl of Lipofectamine 2000 for 12 h before changing medium.

Comet Assay—The neutral comet assay was carried out according to manufacturer’s recommendations (Trevigen, Gaithersburg, MD). Slides were incubated for 30 min in lysis buffer and for 40 min in alkaline solution (pH >13), and comet tails were generated by a 10 min electrophoresis in TBE buffer at 20 V, room temperature. Slides were stained with SYBER Green and DNA migration was analyzed by fluorescence microscopy (Leica DMS 4000B). The tail moment and the tail length were determined using the software package “comet assay II” (Perceptive Instruments, Suffolk, UK). A minimum of 50 cells per experiment was analyzed. All the experiments were done in triplicate.

Protein Extracts and Western Blot Analysis—Protein extracts (total and fractionated) were obtained as described (9). 50 μg (or the indicated amounts) of each extract were electrophoresed on 4–12% SDS-polyacrylamide gradient gel under reducing conditions. Western blot experiments were carried out as described (9). For p-ATM Western blot, proteins were separated on 5% low bis acrylamide gels (acrylamide:bis ratio 5:0.17). The antibodies used and their dilutions were: anti-H2AX (Bethyl Laboratories, Inc., 1:1000); anti-p53 (Ab-1) (Upstate Biotechnology, 10 μg/ml), anti-phospho-p53 (Cell Signaling Technology, 1:1000), anti-Fe65 (9); anti-γH2AX (Ser139) (Cell Signaling Technology, 1:1000); anti-phospho ATM (Ser 1981) (Cell Signaling Technology, 1:1000), anti-APP C-term (A8717 Sigma, 1:4000), anti-Nijmegen breakage syndrome (Santa Cruz Biotechnology, 1:500), anti-lamin B (Santa Cruz Biotechnology, 1:250), anti-β-actin (Sigma, 1:1000), anti-myosin (Santa Cruz Biotechnology, 1:1000), anti-tubulin (Sigma, 1:1000).

RESULTS

Fe65 KO MEFs Are Highly Sensitive to DNA Damage—To explore the functions of Fe65 and of the Fe65/APP complex we generated Fe65 knock-out mice (see supplemental material (part 1)). These mice do not show any obvious phenotypes up to 12 months of age. The most conceivable explanation of these findings is that Fe65L1 and/or Fe65L2 compensate for the absence of Fe65, thus allowing the animal to develop and live normally. However, we explored whether in stressful conditions the amount of Fe65 proteins in Fe65 knock-out cells could be not enough to arrange an adequate cellular response and this could result in a defect that unmask the function of the protein. According to this hypothesis, normal MEFs show a dose dependent increase of DNA damages upon the exposure for 30 min to etoposide or H2O2; comet assay demonstrated that at concentrations of 15 μM, the two agents have only marginal effects (Fig. 1A). On the contrary, three Fe65 KO MEFs derived from three independent embryos showed at these low concentrations high levels of DNA damage (Fig. 1B).

The response of the Fe65KO MEFs to DNA damaging agents was apparently normal as demonstrated by the activation of ATM and by the phosphorylation of Nijmegan breakage syndrome, one of the substrates of ATM (Fig. 1C). The only differences were the higher levels of H2AX histone phosphorylation (γH2AX) and the accumulation of p53 observed in KO MEFs exposed to low doses of VP16 compared with wild type animals (Fig. 1D).

Effects of DNA Damage in Fe65 KO Mice—The differences observed between wild type and Fe65 KO MEFs could imply an abnormal response of Fe65 KO mice to DNA damage. Therefore, we analyzed the sensitivity of our KO mice to x-ray-induced DNA damage. KO male mice of 3 months of age and normal mice, sex and age matched and belonging to the same litter, were exposed to non-fatal, low doses (0.5 and 1 gray) of ionizing radiations. As shown in Fig. 1E, 2.5 h after the exposure to ionizing radiations brain extracts from Fe65 KO mice showed higher levels of phosphorylated γH2AX and a higher
p53 accumulation compared with those of wild type mice. The latter are clear signs of the high sensitivity of Fe65 KO mice to DNA damaging agents.

Fe65 Should Be Present in the Nucleus to Rescue the Hypersensitivity of Fe65 KO MEFs to Genotoxic Stress—It was well demonstrated that the overexpression of Fe65 in different cell lines is accompanied by the accumulation of the protein in the nucleus and APP functions as an extranuclear anchor preventing Fe65 nuclear translocation (8, 15). Furthermore, the COOH-terminal fragment of APP resulting from the cleavage catalyzed by γ-secretase was found in the nucleus associated with Fe65 (16). These observations led to the hypothesis that the processing of APP could be involved in the mechanism regulating Fe65 availability in the nucleus. On the other hand, APP cytodomain is phosphorylated at Thr-668 and this phosphorylation decreases the affinity of APP for Fe65 (26), thus suggesting a second mechanism to regulate the nuclear availability of Fe65. Therefore, we addressed the question of whether the phenotype we observed in the Fe65 KO MEFs is related to Fe65 functions requiring its presence in the nucleus. To test this point we generated two expression vectors encoding Fe65 fused at the COOH terminus to the nuclear export signal of the MAPKK or Fe65 fused to a mutated, nonfunctional, version of this NES, as described previously in Ref. 27. The transfection of the Fe65-NES protein showed no rescue of the phenotype, while the Fe65 fused to the mutant form of the NES had the same effect as the wt Fe65 (see Fig. 2).
The proteolytic processing of APP could be involved in the phenomenon, because of the interaction of APP with Fe65. Therefore, we measured the amount of the APP COOH-terminal fragments (CTF) derived from the cleavage of APP by α- or β-secretase, C83 and C99, respectively. As shown in Fig. 3B, in the wild type, untreated MEFs, only the C83 band is clearly visible; upon the treatment with 40 μM etoposide, the amount of APP-CTF is drastically decreased within few minutes and returned to basal levels 2 h later. This decrease is clearly dependent on the induction of the cleavage of CTF by γ-secretase; in fact, the pretreatment of the MEFs with DAPT, a γ-secretase inhibitor, prevents the observed decrease of APP-CTF (see Fig. 3C). Furthermore, both APP and APP-CTF in cells pretreated with DAPT and exposed to etoposide seem to accumulate up to levels higher than those present in cells treated only with DAPT. These findings suggest a relationship between Fe65 modifications and the cleavage of the APP-CTF. To explore this point we analyzed the cleavage of APP-CTF in Fe65 KO MEFs. As shown in Fig. 3D, in these cells the treatment with etoposide does not induce any changes in the amount of App-CTF, thus further supporting that APP processing is dependent on Fe65 modification induced by genotoxic stress.

DISCUSSION

The phenotype of Fe65 KO cells and mice described in this paper establishes a link between the function of this protein and the cellular machinery devoted to protect from and/or respond to DNA damages.

Previous results suggested that Fe65 could have some nuclear functions, considering its presence in the nucleus and its interaction with several nuclear proteins. These results supported the hypothesis that Fe65 could be involved in the regulation of transcription, although most of them rely on the use of reporter gene assays. Our present results do not exclude this possibility, because dysregulation of specific genes, involved in the control of genome integrity and/or in DNA repair, could significantly contribute to the observed phenotype.

The timing of the modifications of the nuclear Fe65 and of the APP processing we observed in normal MEFs exposed to the genotoxic stress indicates that nuclear Fe65 is modified before (5 and 10 min) the cleavage of APP-CTFs (10, 15, and 20 min). This suggests that the cleavage of APP does not trigger the translocation of Fe65 in the nucleus. On the contrary, it favors the hypothesis that the phosphorylation of APP cytodomain at Thr-668, which decreases the affinity of APP for Fe65, induces the nuclear translocation of a small subset of phosphorylated Fe65 molecules (26). The activation of the cleavage of APP-CTF following the genotoxic stress is clearly dependent on Fe65, because it is completely abolished in cells lacking Fe65. This result confirms that the processing of APP is significantly dependent on its cross-talk with Fe65 (28).

The relationship between the phenotype we observed in Fe65 KO mice with those described in the feh-1 KO worms and in the Fe65; Fe65L1 double KO mice is not obvious (29). In the nematode, the KO of the feh-1 gene leads to an early arrest of the embryonic development in about 50% of the cases (22). The remaining embryos skip this block and proceed to the L1 larval development in about 50% of the cases (22).
stage. This high percentage of surviving embryos suggests that stochastic events contribute to the early block of development. One possibility to be explored is that KO embryos have an increased sensitivity to DNA damage, thus in many cases they do not survive up to the larval stages. This hypothesis fits with the notion that DNA-damage-induced checkpoint is silenced during early stages of embryo development (30) to ensure that cell cycle progression is not hampered by DNA damage. In the Fe65;Fe65L1 double KO mice the phenotype observed (23), mainly characterized by focal heterotopias of neurons likely due to the pial basement membrane disruption and intriguingly similar to that observed in APP;APLP1;APLP2 mice (24), is very likely due to the impairment of an unknown Fe65 function different from that responsible for the phenotype we are describing. However, it should be considered that numerous human genetic diseases due to mutations of the DNA damage response machinery are also characterized by marked neurological defects (31). This close association has been interpreted either by hypothesizing a particular sensitivity of the neuronal cells to DNA damage or to the possibility that proteins involved in the response to DNA damage and cell cycle control also have additional and distinct functions in neurons.

The possible relevance of these results to the pathogenesis of AD remains to be addressed. There are several observations that suggest exploring whether an altered processing of APP, and in turn a modification of its functions, could negatively affect the response of the neurons to DNA damage in AD brain, thus leading to chronic accumulation of DNA damages that could significantly contribute to the neuronal loss characteristics of AD pathology. In agreement with this possibility, it was reported that AD brains show high incidence of nuclei containing DNA strand breaks (32) and fibroblasts and lymphocytes from AD patients, exposed to DNA damaging agents, show more DNA strand breaks than the cells from normal subjects (33). Second, it should be taken into account that numerous results indicate that the neurotoxic effects of β-amyloid peptides could be dependent on its prooxidant activity (34), which could favor oxidative DNA damage. Third, an altered response of neurons to DNA damage in AD is also suggested by the reexpression of cell cycle proteins observed in AD brain (for a review, see Ref. 35). This reexpression occurs a long time before the death of the neuron, thus it is hard to be considered part of a suicide mechanism to eliminate damaged cells. On the contrary, it could represent part of a mechanism to protect the cell by favoring the repair of DNA damages, also considering that cell cycle reentry also occurs in neurons treated with DNA damaging agents (36).

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